abstracts: poster presentations

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Science Education: Basic Science Research

P1/B1

Extracurricular Biology Research in a High School Setting.

A. Krishnan, C. Chopra, S. Flynn, R. Gupta, G. Pereira, R. Sabherwal, S. Tyson, M. Fields; Sidwell Friends School, Washington, DC.

Students at Sidwell Friends School in Washington, D.C. have the opportunity to conduct original research both inside and outside of the classroom. If a student wants to do research after completing one year of Biology they can join the BRAIN (Biological Research and Investigations in Neuroscience) Club or partner with scientists from other research institutions. Extracurricular research enables students to employ research techniques they have gained through science classes in lab-like settings. This year, students chose to conduct experiments *Danio rerio*, or zebrafish. Their projects include: (i) Vision is vital to many animals and some have mechanisms to repair damaged retinas. We tested how Gamma-Amino Butyric Acid (GABA), a neurotransmitter known for calming nervous activity, would affect that ability. Blinded zebrafish were exposed to various concentrations of GABA and their recovery was monitored. We found high levels of GABA blocked the regeneration of damaged retinas, often stopping regeneration triggered by secreted growth factors. (ii) Fluoxetine (Prozac) is a selective serotonin reuptake inhibitor (SSRI) and a commonly-prescribed antidepressants. Though acute fluoxetine is rarely, if ever, administered in humans, acute treatment of zebrafish mirrors the “lag period” of SSRI effectiveness in humans. As such, this experiment aims to study the differing behavioral effects of chronic and acute fluoxetine on novel tank diving behavior in *Danio rerio*. (iii) We are testing the effects of Ethanol on adult Zebrafish motor skills. One group will be exposed to a 1% Ethanol solution, one group will be exposed to a 2% solution, one group will be exposed to a 3% solution, and one group will not be exposed. In conclusion, we have found that the group exposed to the 3% solution exhibited the largest decrease in motor skills. (vi) We will quantify the effects of sugar and artificial sweetener on the development of zebrafish embryos. Expected results include deformities and defects in the head area and death (aspartame), as well as obesity and accelerated heart rate (sugar). We will conduct this experiment by creating varying concentrations of sugar and aspartame. We will quantify our results using Image J to measure spinal curvature and any defects we see, and recording embryo death. (v) the biodegradability, biocompatibility, and minimal immunogenicity makes collagen a valuable biomaterial. For each application of collagen, the properties of the collagen need to be optimized. In this study, the length of the de-hydrothermal treatment and the materials morphology were varied to characterize the effect of the material processing and morphology on the mechanical properties and in vitro collagenase resistance of the collagen matrices.

P2/B2

Introductory Biology Research in a High School Setting.

A. Krishnan, V. Chuadhry, A. Donnelly, R. Gupta, **A. Krishnan**, M. McGraw, A. Oswald, A. Sabherwal, A. Selassie, E. Yu, M. Fields; Sidwell Friends School, Washington, DC.

Students at Sidwell Friends School in Washington, D.C. have the opportunity to conduct original research both inside and outside of the classroom. If a student wants to do research after completing one year of Biology they can join the BRAIN (Biological Research and Investigations in Neuroscience) Club or partner with scientists from other research institutions. Extracurricular research enables

students to employ research techniques they have gained through science classes in lab-like settings. This year, students chose to conduct experiments *Danio rerio*, or zebrafish. Their projects include: (i) in this experiment, we observed the effect of nitrites and nitrates on zebrafish embryos. We tested a total of 6 groups of 25 zebrafish embryos each: one low concentration of 100 mg/L and one high concentration of 300 mg/L for both nitrites and nitrates as well as two control groups. We analyzed the teratogenous effects of nitrogen-based runoff and fertilizers and how these factors contribute to the development of pericardial edemas and axial malformations. (ii) in our experiment, we are observing the effects of elevated glucose exposure on the development of zebrafish embryos. This is similar to gestational diabetes, where the fetus experiences various harmful effects because of the early exposure to elevated glucose. Zebrafish embryos will be placed in regular embryo water, 0.5% glucose solution, 0.25% solution and 0.1% solution for 4 days. We are testing for mortality rate, yolk-sack size and weight. (iii) One of the most pressing issues today is pollution, specifically of our oceans. The sun causes plastics, such as polyethylene terephthalate (PET), to break down, releasing toxic chemicals into the water. This causes zebrafish embryos to hatch later, have increased mortality, and demonstrate abnormal characteristics. Our experiment mimics the effect of plastic contamination on aquatic life and highlights the damage of human pollution. (iv) It is commonly perceived that e-cigarette fluid is a safer alternative to traditional cigarettes. We were interested in comparing the effects of both substances on developing zebrafish. We exposed the embryos to different concentrations of nicotine and e-cigarette fluid. We found that the group exposed to e-cigarette fluid developed more axial malformations which resulted in a decreased capacity for fine motor skills as well as a higher mortality rate. (v) Roundup is used by millions of people every day as the world's most popular herbicide. Recently there has been controversy surrounding the effects of the herbicide. Improper application can lead to cancer and other diseases. This experiment tests whether RoundUp can cause motor system failures.

P3/B3

Computational Comparative Genome an alysis of Salmonella Enterica Subsp Enterica Serovar Reading.

S. Kang[1], D. Kim[1], S. Baek[1], S. Kim[2], S. Chung[2]; [1]Johns Hopkins University, Baltimore, MD, [2]FTI, Rockville, MD.

Salmonella enterica subsp. *enterica* serovar Reading is a multidrug-resistant food-borne pathogen of which infections in human have accounted for serious illness including mortality. Genome sequences of seven hundred and ninety-nine *Salmonella* Reading isolated from 27 resources in 11 countries at 21 year-points were downloaded from National Center for Biotechnology Information database. Single nucleotide polymorphisms profile was constructed using Parsnp and the evolutionary history was inferred using the Maximum Parsimony method with 100 bootstraps on MegaX. Antimicrobial resistance genes in each genome were identified using AMRFinder with NCBI's Bacterial antimicrobial Resistance Reference Gene Database. Prophage and virulence factors were identified using blast with prophage and virus DB from PHAST and *Salmonella* VF database from Virulence Factors of Pathogenic Bacteria, respectively. *Salmonella* Reading were clustered to 7 clades and phylogenomic differences were likely correlated to countries and sources. Antimicrobial resistance genes abundance differed between countries while virulence factors and prophage were similar in all genomes.

P4/B4

Enhancement of Power Generation in Microbial Fuel Cell through Supplementation of Platycodon Grandiflorus Roots.

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The current study reports the evidence of enhancement in power generation from cellulosic biomass in microbial fuel cell (MFC) systems by supplementing Chinese bellflower (*Platycodon grandifloras*) roots powder. Mediator-less two chamber H-type MFCs were prepared using rumen fluid as anode inocula to convert finely ground pine tree (Avicel) at 2% (w/v) to electricity. Dried bellflower roots were ground to pass 1mm sieve and added to the anode of MFC at 0.1% w/v dosage for treatment. MFC power and current across an external resistor were measured daily for 10d. At the end of incubation on d10, collected gases were measured for total gas volume and analyzed for gas composition on gas chromatography. Supplementation of bellflower roots powder to MFC anode chamber increased power generation and CO_2 production. Over the 10d experimental period, power density normalized to anode surface area were between 17.0 and 37.7 with average of 32.5 mW/m^2 in bellflower MFCs, and between 16.8 and 19.8 with average of 18.2 mW/m^2 in control group. These observations imply that Chinese bellflower root components would alter microbial fermentation of cellulose compounds favorable to produce bioenergy efficiently in MFC.

P5/B5

Transportation of Human Primary Hepatocytes for Transplantation.

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Cells preserved in dry ice, liquid nitrogen or live cells in complete medium are current methods used for cell shipments. These methods require special handling and there is limited survival time for cells during the transportation process. Here we report a novel shipping procedure that is inexpensive, environmentally superior and protects cell viability and integrity at ambient temperature. We prepared mammalian cells using Cell Transportation Matrix (CTM™) with protocol provided by the manufacture and kept the cells at room temperature (RT) for 7 days. Cells maintained in CTM™ at room temperature for 7 days have greater than 80% viability, and retained full biological functions. All together, unlike dry ice and liquid media, CTM™ shipping is a novel way to transfer and ship cells with positive economic, practical and environmental implications.

P6/B6

A Semester-long Undergraduate Molecular and Cellular Biology Laboratory Project.

K. Chambers; Concord University, Athens, WV.

A laboratory curriculum was developed to introduce undergraduate molecular and cell biology students to multiple techniques commonly used in the field in the context of an extended laboratory project. Over the course of the semester, the students clone the human α-tubulin (TUBA1B) cDNA into a green fluorescent protein mammalian expression vector (pEGFP) and express it in mammalian cells. The localization of the fusion protein is then visualized using fluorescence microscopy. Using this fluorescently-labelled recombinant protein, students then observe the effects of nocodazole on microtubule networks in cells. Finally, students complete an exercise that exposes them to the field of

bioinformatics. As this project is part of an undergraduate course, it is designed for all skill levels and is accessible for numerous students. It allows students at a small liberal arts institution to acquire skills in many molecular and cell biology experimental techniques and gain a research-like experience. The project provides students with a conceptual understanding of various techniques and how they can be used in conjunction to answer scientific questions. Additionally, students develop analytical and critical-thinking skills. Each week students interpret data and assess the reliability of their results. Unlike traditional "cookbook" labs, students are asked to recall and relate data from week to week over the course of the entire semester. At the end of the semester, students communicate their results in a conference-like poster presentation and discipline-appropriate scientific paper.

P7/B7

Incorporation of Crispr/cas9 into a Semester-long Investigative Lab on the Cell Biology Of*C. Elegans*.
G. De La Torre Pinedo, S. K. Olson; Pomona College, Claremont, CA.

Course-based undergraduate research experiences (CUREs) provide authentic research opportunities in a classroom setting, allowing greater numbers of students to be exposed to the scientific process and to truly take ownership of their projects, which in turn encourages more students to pursue careers in science. For the past few years, we have incorporated CRISPR/Cas9 technology into the lab component of an Advanced Cell Biology class to introduce undergraduate students to this cutting-edge technology. Students design CRISPR-based independent projects that explore various facets of C. *elegans* eggshell assembly, which is the focus of our research lab. Thus, students generate novel data that furthers our lab's research agenda. Several different CRISPR approaches are commonly used in the C. elegans community, so we explored 3 different methods to find the one that best balances successful edits, time, cost, and pedagogical goals. First, students in the Fall 2017 class used the streamlined and modular SapTrap method (Schwarz & Jorgensen) to modify their chosen protein with fluorescent tags, affinity tags, and degrons. With this method, 6/8 groups successfully cloned their constructs, 4/8 saw evidence of genome edits, and 1/8 were able to perform their proposed experiments. In Summer 2019, a group of high school students spent 4 weeks learning about CRISPR and working on a plasmid-based method that incorporates PCR, cloning, and selectable markers (Dickinson & Goldstein). The students successfully generated the DNA building blocks, but the session ended as they were ready to assemble their vector. In Fall 2019, Advanced Cell undergraduate students used a co-CRISPR approach (Paix & Seydoux, Arribere & Fire), where they had the option of fluorescently-tagging their protein or generating a mutant. Students designed the guide RNA and homology-directed repair oligos, and screened for edits in injected worms. They spent the remainder of the semester studying their edited worms to explore localization patterns, phenotypic changes, and/or protein interactors, with the overall goal of understanding the role of their protein in C. *elegans* eggshell formation. Six of the 8 groups had successful genome edits within 4 weeks and were able to conduct their independent studies. The most recent approach seems the most promising for efficiency, but is limiting in the number of edits that can be made with one injection, and is significantly more costly than the plasmid-based approaches. Ongoing efforts aim to reduce the amount of work outside of class hours and to allow for more flexibility in edit designs.

P8/B8

Characterization of Novel Immortalized Mouse Renal Proximal Tubular Epithelial Cells.

M. Schmidt[1], K. Otterpohl[2], B. Busselman[2], I. Chandrasekar[2]; [1]NSF-REU summer student from Drake University, Sanford Research, Sioux Falls, SD, [2]Sanford Research, Sioux Falls, SD.

Nonmuscle myosin 2 (NM2) play critical roles in cell adhesion, migration, and cell division. Previously, we also demonstrated NM2's role in clathrin-mediated endocytosis (Chandrasekar et al; 2014). In order to further study the role of NM2 in cellular transport pathways, our lab uses mouse kidney and renal epithelial cells as a model system. My research directly dealt with mouse proximal convoluted tubule (PCT) epithelial cells generated from conditional knockout mouse model of the *Myh10* (NM2B) gene. These mice were crossed with a temperature sensitive, immorto SV-40 antigen +/Tg mouse in order to immortalize the cells. The goal of my summer project was to characterize the PCT *Myh10* cKO cell line and confirm its applicability as an in *vitro* research tool. The PCT *Myh10* cKO cells were cultured on trans-well polyester membranes for 2 days at the permissive 33C, to enable cell proliferation and then moved to 37C on an orbital shaker to mimic fluid shear stress experienced by renal cells and to facilitate maturation and polarization for up to 6-8 days. Some membranes were treated with doxycycline for 48 hours to initiate the *Myh10 gene* knockout. The cells were fixed and stained using immunofluorescence methods or made into lysates for western blots in order to identify the localization and expression patterns of proteins specific to PCT cells. Many treatments including a fibronectin coating of the membrane, 5%, 20%, 60% collagen coat, and lysophosphatidic acid (LPA) were used to promote polarization of the PCT cells.Immunofluorescence confocal microscopy revealed that large pockets of cells in culture were polarized and grew to 5-8 µm in height in the 20% collagen coated group. Also, through western blotting techniques and immunofluorescence methods knockout of Myh10 protein was confirmed. Proteins specific to PCT cells including megalin (apical endocytic receptor) and SGLT2 (Na^+-sodium cotransporter) were visualized by immunofluorescence or analyzed by western blotting techniques. These cells will serve as novel renal epithelial cell models to facilitate our understanding of receptor mediated transport pathways unique to proximal tubular epithelial cells and its role in maintaining kidney function.

Science Education: Innovations in Undergraduate Teaching

P9/B9

Investigating Relationships among the Individual, the Team, Personal Strengths, and Peer Evaluation in a Team-based Introductory Biology Course.

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Team-based learning is an effective way to learn in STEM. We examined the relationships of team scores and individual scores and whether sex or personal strengths affect individual scores or team scores. In the team-based introductory biology course for majors we studied, semester-long teams collaborate daily on in-class activities, take weekly quizzes, and work on a semester long project. To encourage development of team skills and personal accountability, every week student roles on the team rotate and team members note each other's contributions. At the end of the semester students evaluate teammates' contributions. We collected data from 357 students in three course sections and analyzed elements of individual and team scores. To achieve a high degree of diversity on teams, we sorted

students based on apparent race and sex (from student pictures on course registration lists), honors status, geographic and cultural diversity, and personal strengths. We based geographic and cultural diversity on students' first language and on where they attended high school, reported on a pre-class survey. An other survey question asked them to identify a personal strength that we matched to the categories (Administrator, Artist, Communicator, or Expeditor) in Wright and Boggs 2003. At the end of the semester, team members indicated who exhibited traits characteristic of each personal strength category. • Individual and team scores were not correlated. • 23% of students earned a team score that negatively affected their final course grade percentage; only 1% of students earned a team score that negatively affected their final letter grade. • Men and women had no significant difference in scores. • Students identified by peers as Administrators and Expeditors have higher individual and peer evaluation scores. • Peer-evaluation score and team score were not correlated. • Peer-evaluation score and individual score were positively correlated. • Peer-evaluation scores were not different between men and women. • All teams identified individuals who contributed to teamwork according to each personal strength category. • Self-identified personal strength and team-identified personal strength often did not correspond. We conclude that a wide variety of students can be successful in this course, which emphasizes personal accountability and team skill development.

P10/B10

The Use of an Inquiry-based Laboratory Teaching Approach Using a Biofuels Assay.

F. Norflus; Clayton State University, Morrow, GA.

In order to engage students in biology laboratory classes, teachers attempt to make the material relevant and use an inquiry-based approach where students are responsible for designing their own experiments. This project discusses how the Bio-Rad biofuels kit (catalogue #1665035EDU) was used in a biotechnology class to show a real world example of biotechnology but also to teach students about designing experiments and interpreting data. Students were able to pick a parameter to test and also needed to trouble shoot the experimental details at certain points. The basics of this experiment is that the enzyme cellulase breaks down cellulose, found in plant cell walls, into glucose which can be further converted into ethanol during the process of fermentation. Since the enzyme cellulase is found in a few organisms including plants and fungi, this system could ultimately be used to develop biofuels as an alternate source of energy. In the class experiment, an artificial system was used. The enzyme cellobiase converts the artificial colorless substrate p - nitrophenyl glucopyranoside into the yellow colored product p-nitrophenol. The company provides purified cellobiase enzyme but it can also be obtained from an extract of mushrooms. Students used the company provided enzyme as well as extracts of different mushrooms and different parts of the mushrooms to determine which produced the most amount of cellobiase. Sample student data using enoki and shitaki mushrooms will be presented as well as suggestions on where students had difficulties with the experimental details and data analysis. The data shows that the mushroom extracts had greater enzyme activity than the purified enzyme provided by the company.

P11/B11

A Small Scale Novel Experimental Design Assignment as a Precursor to Cures Using Zebrafish as a Model System.

A. Dasgupta, S. Sarmah, J. Marrs, K. Marrs; IUPUI, Indianapolis, IN.

Scientific literacy or the ability to apply the processes of science was identified as one of six competencies outlined by Vision & Change (AAAS, 2009). The experimental design is a core scientific ability needed for a deep understanding of how biological knowledge is generated and gives students experience to formulate a plan to address a scientific question. Studies showed that early immersion into discovery-based research experiences, like experimental design, engages student during their undergraduate education (Elgin et al, 2016). One way to expose students to experimental design is participation in course-based undergraduate experiences (CUREs) (Dolan, 2016). While CUREs are an effective way to introduce a research problem to large student numbers, faculty might initially like to try small scale inquiry-based lab modules and gradually progress towards developing a CURE. In this project, we presented fall 2018 introductory biology students an opportunity to practice experimental design with an original lab module. The module centers on designing an experiment to study the effects of environmental toxins using zebrafish. Our assessment design focused on the following criteria: short term implementation; personal relevance to students; efficient use of limited resources; probing students' quantitative skills; open-ended with easy scoring for 300-600 students. With the aforementioned criteria, the module was organized to cover 2 lab sessions with 3 components namely: pre-reading, experience handling and imaging live and preserved zebrafish, and the experimental design exercise. Pre-reading, intended to level prior knowledge differences, involved primary literature readings to gauge the societal relevance of certain toxins, like alcohol and nicotine. Next, students were familiarized with the wild type and chemically treated zebrafish morphology differences in the lab. Last, students completed the experimental design assessment starting with the prompt: **How would you design a simple experiment to determine the effects of a particular compound of interest on zebrafish development or behavior?** Aligned with the basic competencies of experimentation, students were required to cover various aspects such as framing testable hypotheses, identifying experimental variables and prediction of experimental outcomes. Student responses were scored using the EDAT (Sirum & Humburg, 2011) and RED rubrics (Dasgupta et al., 2014). Our findings indicated the most common difficulties were manipulation of variables, measurement of outcome, and accounting for variability in the proposed hypothetical experimental plans. Positive outcomes regarding students' satisfaction with the modules is encouraging for us to continue implementation and assessment.

P12/B12

Effects of Guided Practice in Drawing on Model-based Reasoning and Use of Drawing for Studying.

J. R. Burns, P. D. Heideman; William & Mary, Williamsburg, VA.

Model building can use drawing or sketching (D/S) as a mechanism to help the drawer learn information (study), solve problems (model-based reasoning, MBR), and communicate. Unfortunately, many students fail to master D/S skills due to the effort and instruction required. We applied guided practice of D/S to an undergraduate first-semester Introductory Biology majors course, which focuses on the cellular and molecular basics. Using a combination of surveys and semi-structured interviews, we aimed to assess (1) how much students continue to use D/S as a study tool in subsequent semesters, (2) the extent of D/S usage for MBR, and (3) whether the use of D/S was correlated with correct answers on

exams. Students decreased their use of passive study methods during the course (pre: 56% to post: 37%; $P < 0.01$). Major changes included less visual review (from 38% to 25% of study time; $P < 0.01$) in studying and more D/S (pre: 13% to post: 42%; $P < 0.01$). In the pre-survey, only 5% of students listed D/S as part of their 'most effective' study method, rising to 33% in the post-survey ($P < 0.01$). Students who reported reluctance to change their study methods used less D/S while studying than those willing to change their study methods. During the class that assigned D/S for homework, over 90% of students interviewed used D/S when answering questions on assessments that did not require D/S, but < 1/3 used D/S for MBR. The following semester, when D/S was no longer taught in class, less than 20% used D/S on questions that did not require D/S. Data collection and analysis are ongoing for (2) and (3) above. These outcomes will be reassessed yearly. The results at the end of the first semester are unsurprising, as students were encouraged to draw for homework and exams. An important question for ongoing data collection is whether students continue to use D/S in subsequent semesters and in courses that neither require nor encourage drawing to learn. Our preliminary conclusion is that a course in this format can support development of D/S for MBR while developing more active study methods.

P13/B13

Engaging Students through Forensic Sciences and Using the Class-Bio Survey to Assess Student Attitudes and Beliefs About Learning in General Biology.

C. Jones; Lane College, Jackson, TN.

Lane College is a historically black college committed to educating underserved minority students. My objective was to give students a diverse teaching experience and improve student success in General Biology I by engaging students through forensic sciences. The study of forensic science has increased in popularity and given teachers a technique to teach biology, chemistry, and physics. I implemented forensic science in the laboratory in one of my two sections of General Biology I in the Fall 2018 semester. Students studied and determined the items that needed to be analyzed from a mock crime scene after being presented some basic forensic science techniques in the first week of laboratory. Each additional week the students participated in hair analysis, fiber analysis, soil analysis, synthetic blood tests, chromatography, and DNA fingerprinting. To measure the success of the intervention, I used CLASS-Bio Survey to assess student attitudes and beliefs about Learning (n=32) that was given pre and post to both sections as a way to compare the effect of the intervention. The pre survey perceptions were higher for the section that I was giving the Forensic Science intervention in all categories Enjoyment, Real World, Problem Solving Synthesis and Application, Problem Solving Effort, Conceptual, Problem Solving Strategies, and Reasoning. At the end of the course, the class that received the forensic science intervention had a 19.1% greater increase in Enjoyment, 15.9 % greater increase in Problem Solving Effort, and an 18.4 % greater increase in Problem Solving Strategies compared with the class that did not receive the intervention.

P14/B14

The Genomics Education Partnership: New Science Projects Expand the Opportunities for Student Participation in Genomics Research on the Evolution of Parasitoid Wasp Venoms and Insulin Pathways Genes.

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The Genomics Education Partnership (GEP) started in 2006 to introduce genomics in undergraduate classrooms/labs. Genomics provided an ideal means to involve large classes in authentic research with benefits similar to the wet-lab apprenticeship model, and at a low cost. The original scientific questions focused on the evolution of *Drosophila*'s Muller F element (dot chromosome) across multiple *Drosophila* species. Students at institutions ranging from community colleges to tier-1 research universities participate in improving the genome sequence and annotating the mostly heterochromatic F element and a euchromatic region near the base of the D element in different *Drosophila* species. These improved sequences and gene annotations have been used in four scientific publications. The GEP has grown to a network of more than 100 colleges and universities. To support future growth, the GEP has evolved from a centralized leadership model to a distributed leadership model, and has introduced two additional science projects: parasitoid wasp venom and biological pathway gene annotations. The parasitoid wasp venom annotation project aims to provide insights into the functions and evolution of venom genes. Current research has hinted at putative mechanisms of action, and it is expected that the improved annotation of venom genes produced by GEP students will help to characterize the functions of venom proteins, and to identify proteins with potential dominant negative functions. Similarly, understanding the genomic context of venom genes should help resolve questions concerning the evolution of venom genes. The pathways project uses network analysis approaches to elucidate the evolution and function of biological pathways, initially focusing on the insulin signaling pathway. Past studies have shown that the rates of evolution for the gene's protein-coding regions are correlated with its position within the network, the number of physical interactions, expression levels, and the existence of closely-related paralogs. GEP students will produce gene annotations for the putative orthologs and paralogs of *D. melanogaster* genes involved in the insulin signaling pathway in 27 *Drosophila* species. These improved gene models will be used to ascertain if the rates of evolution for the genes' regulatory regions parallel that of their protein-coding regions. These new opportunities expand both the range of scientific questions in which students may find particular interests, as well as the possibilities of pairing annotation projects with wet lab investigations. GEP is currently recruiting new members (http://gep.wustl.edu/help/contact_us). Supported by NSF IUSE-1431407 to Sarah C.R. Elgin, NIH 1R35GM133760 to NTM, NSF IUSE-1915544 to LKR, and NIH IPERT-1R25GM130517-01 to LKR.

P15/B15

Compass "Creating Opportunities for Students in Sciences" Program Heightens Awareness of Careers in Science for Underrepresented Students in Rural North Carolina.

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The University of North Carolina at Pembroke (UNCP) was awarded an S-STEM program (*COMPASS: Creating Opportunities for Students in Sciences* (award #1356582, 2014-2019, $618,993). The program enrolled 27 talented students (3 staggered cohorts of 9 students each) with demonstrated financial need majoring in Biology (16), Chemistry (5), double Bio/Chem (4), and Biotechnology (2). The participant group (4 males and 23 females) was very diverse (36% Native-American, 32% African-American, 3% Latino, 14% Middle-East, 4% Asian-American, 11% white). Students received up to $6,000 a year from their sophomore to senior year. The program successfully met the goals of retaining and graduating students in a timely manner, preparing them successfully for careers in STEM through academic and career support programs, and fostering a community of faculty mentors and scholars. COMPASS scholars exceeded retention and 4-year graduation rates for general STEM students at UNCP (which were only 48% and 18.7% respectively at the start of our program). Twenty-five of the 27 scholars (92.6%) graduated in 4 years. One cohort III student graduated in 3.5 years, one graduated in 5 years because of a change of path. The program achieved an outstanding record of retention and graduation and COMPASS graduates demonstrated their growth when pursuing jobs and graduate school opportunities, including 64.7% of students who graduated with honors. Presently 41% of the students are in STEM related areas (PhD, masters, post-bac, teaching science or industry), many of them in NC. It became apparent to us that our talented students in Biology and Chemistry degrees overwhelmingly have goals of holding health related occupations, mainly MD, PharmD, PA, PT, and DVM. The program has been transformative for the lives and careers of these scholars and even those who persist in their plans to join a professional school recognize that COMPASS opened their eyes to careers and possibilities they did not know existed. In fact, the exposure to careers through site visits and conversations with STEM professionals was one of the most student-cited benefits of the program. Attending and presenting at professional conferences was also valuable and helped student's network and foster a sense of belonging in STEM. Being exposed to research experiences (77% participating) early on has also contributed to their openness to science careers. The community aspect is without a doubt the most valuable overall to all scholars. They all agreed that the program enhanced their sense of belonging to UNCP, and that the support of the COMPASS fellows was important to their academic success. The figure of the mentor not only was a source of support and integration within the program, but provided an additional source for academic advice and career support.

P16/B16

Enduring Use of Learner-generated Drawing to Learn Biology: Brief Intervention Versus Extended Practice.

P. D. Heideman, L. Wilkin; William & Mary, Williamsburg, VA.

Learner-generated drawings are useful for comprehension and model-based reasoning (MBR), but students struggle to represent biological structures, concepts, or hypotheses by drawing and sketching (D/S). Recent reviews emphasize that drawing and sketching as a skill is difficult to teach and difficult for students to learn. In this study, our objective was to test two approaches to develop enduring use of learner-generated drawing for studying and MBR. We hypothesized that students taught a method to

develop drawings that quickly represent structures or sequences of events might lead to enduring use of learner-generated D/S. In a prior study, we reported that a short D/S intervention with first-semester biology students improved recall and problem solving. In addition, 5 months after the intervention, students in the intervention reported increased use of D/S for studying (effect size = 0.67). Here, we ask (A) whether gains of D/S for studying were maintained for a longer duration, and (B) whether semester-long courses that taught, modeled, and encouraged D/S for comprehension and MBR might have similar or greater effects. The results showed (A) that gains from the short intervention were not maintained: in follow-up surveys conducted 2.5 and 3.5 years after intervention, participants matched the comparison group in D/S. (B) However, students who had taken 1-2 biology courses incorporating extensive instruction and applications of D/S for MBR reported, 1-2 years later, more use of D/S for studying than those in a comparison group (after 2 D/S courses: 23% of study time, and with no D/S courses: 6% of study time; effect size > 1) and were more likely to refer to drawing when describing their 'most important study method' (with no D/S courses:10%, and after 2 D/S courses: 52%). One-two years after their second D/S course, students reported using D/S in a median of 3 courses. A single D/S course had intermediate values for these measures. We conclude that a short intervention may be insufficient for enduring changes in use of D/S for studying, while engagement of students with D/S for MBR in semester-long courses may produce lasting changes in study behavior.

P17/B17

Drawing for Model-Based Reasoning: Problem-Solving and Performance on Exams in Integrative Biology and Cell Biology.

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Drawing and sketching (D/S) are well established as tools for learning and model-based reasoning (MBR), but students struggle to apply D/S for study and reasoning. We hypothesized that instruction in D/S with feedback and regular application in MBR and hypothesis testing would prepare students for MBR in subsequent courses. In a major's lecture course in integrative biology of animals (N = 150 students), we incorporated D/S for MBR. Materials included a conventional textbook, printed notes from the instructor, D/S instructional videos, and sketch uploads as homework. After exams, qualitative "think-aloud" interviews asked if and how students applied sketches to solve problems. During the integrative biology course, most students (80%) used sketches as a memory recall tool (e.g., 'a mental image'), just over half (57%) used sketches as an MBR tool when solving transfer problems on exams. The use of D/S was related to success on exam problems: for the answers analyzed, 81% of those receiving full credit had involved a sketch as a reasoning tool, while only 24% of answers receiving less than 2/3 credit involved a sketch as a reasoning tool. In a post-survey on usefulness of course materials, the second-most highly rated were D/S instructional videos. In a later cell and molecular biology course, students who had received extensive D/S instruction applied D/S both for studying and for MBR on exams. The results indicate that students given D/S instruction adopted sketching as a tool for learning, retrieval practice, and self-evaluation, and many successfully used sketches as mental models to solve difficult exam problems. We conclude that D/S can be incorporated into a large undergraduate course to develop MBR skills in biology students, and that students who received instruction continued applying D/S for MBR in a later course in molecular cell biology.

P18/B18

Analyzing Student Performance and Problem-solving Barriers in Open-ended Genetic Problems.

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The need to build problem solving skills in STEM undergraduates has been widely reported. This study is part of a larger multidisciplinary investigation seeking to better understand undergraduates' STEM problem solving performance to develop a model that will guide instructional support of students' problem solving skill development. Specifically, the project is investigating the role of student knowledge structure, knowledge retention, and barriers in solving open-ended genetic problems. Study participants were enrolled in an upper-division genetics course that covers transmission, molecular, and applied genetics. This four unit course consists of two 110 minute lectures and an optional 50 minute discussion session each week of a 10-week quarter. 95% of the 293 enrolled students were majoring in STEM and required to take the course. To gather a baseline of students' genetics knowledge, ta Genetics Diagnostic Test (GDT) was administered to students in the first and last week of class. Students were also asked to complete an online Metacognitive Awareness Inventory (MAI) the first and last week of class. In addition to the CADEK and MAI, students answered open ended genetic problems on an in-class summative assessment, from which ten high performing and ten low performing students were identified and invited to participate in one hour think aloud interviews (TAInt). The TAInt were conducted during weeks 7 and 8 of the quarter and entailed participants speaking through their thought processes while solving several open ended genetic linkage problems. To assess levels of knowledge retention, participants completed a follow up TAInt 8-9 weeks after the end of course during which they solved the same open ended problems. Several developed instruments were used to analyze participant responses on the open ended genetic problems from the in-class assessment and transcribed TAInt. Specifically, the COSINE (Coding System for Investigating Sub-problems and the Network) method is an in-depth analysis of the difficulties students have during the problem solving process. To apply the COSINE method, sub-problems that correlate with specific steps of genetic linkage problems are assigned a code based on student performance on a particular task. Quantitative metrics are being developed based on resulting codes to provide insight into where and why students are unsuccessful, in addition to correlating student performance with incoming genetic knowledge, misconceptions, and metacognitive level.

P19/B19

Integrating Mathematics and Biology in the Classroom: a Compendium of Case Studies and Labs.

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We developed a compendium of exercises, cases and wet labs to support a mathematics of biology course that brings together undergraduate students from biology and the mathematical sciences. This book contains background exercises and case studies with real data and experimental labs that iterate through experimental design, data collection, analysis, and follow-up experiments. Difference equations and differential equations are introduced along with an R tutorial and embedded code throughout the text using examples from all levels of biological organization. At the level of cellular biology, we offer several exercises including tumor growth, enzyme function, an immune response, and quorum sensing. For the case on quorum sensing, we introduce the basic biology and system of autoinducers and response via the *lux* genes leading to phenotypic changes in the bacterial population. We then formulate a model of the sequential steps starting with bacterial population growth to the up-regulation of the *lux* gene, protein expression and the feed-forward responses. We explore the switch-like response to the

autoinducer through fitting the model to data from *Aliivibrio fischeri* using some known parameters and fitting others. Sensitivity analysis reveals the parameters with the greatest effects on response. Finally, we explore temporal aspects of the model and pose further biologically relevant questions for students to explore. The preparatory exercises, cases such as this one, and experimental labs together engender confidence in the ability of each student to address questions, both experimentally and mathematically, and highlight the value of working in interdisciplinary teams.

P20/B20

Sustained Mentorship Promotes the Development of Active Learning Strategies in Undergraduate Biology Classrooms: Evidence Gained from the Promoting Active Learning (PALM) Network.

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Here we report on the work of the Promoting Active Learning and Mentoring Network (PALM), a National Science Foundation-supported project started in 2016 and dedicated to leveraging the expertise found in life science professional societies to provide long-term mentorship in developing active learning techniques in the classroom. PALM mentees work one-on-one with mentors that are seasoned in active learning strategies and travel to their mentor's institution to observe classes, plan modifications for their own classes, and strategize the best active learning techniques that are suited to their class' particular needs. A monthly online journal club and regular opportunities to meet in conjunction with the Society for the Advancement of Biology Education Research (SABER) and ASCB meetings provide a dynamic teaching and learning community for mentees and mentors alike. After their mentorship, mentees are encouraged to work with their colleagues on active learning strategies and to become a PALM mentor to further the spread of effective active learning strategies to an ever-wider audience. We have assessed the efficacy of PALM mentorship through the use of the Classroom Observation Protocol for Undergraduate STEM (COPUS) protocol (Smith *et al.*, 2013) on pre-mentorship and post-mentorship videos of mentee teaching. The COPUS results have revealed a consistent shift in mentee teaching practices from a strict focus on lecture to a more diffuse combination of classroom activities in which the student is more directly active and engaged in the class and the instructor is more directly engaging students in a more personalized manner. While the specific changes in classroom practice vary between individual mentees due to the distinct needs and environments of each class, there is a consistent picture of more student-centered classrooms that are more engaging and dynamic for both student and instructor. Overall, the COPUS data suggests that the long-term mentoring strategies brought forth by PALM are an effective and sustainable means to promote the development of active learning techniques among undergraduate biology instructors.

P21/B21

Mentorship in Converting Research into a CURE: the Mentoring the Integration of Research into the Curriculum (MIRIC) Initiative.

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The American Association for the Advancement of Science (AAAS) *Vision and Change* document has spurred several initiatives designed to improve the way in which undergraduates learn science. Often,

these initiatives have been disseminated as one-time workshops that generate awareness of and interest in developing authentic research experiences for undergraduate STEM classrooms. Conversely, whole "standardized" curricula have been developed and made available for adoption. However, successfully generating the sustainable change necessary to bring real reform to undergraduate science education should benefit both students and faculty scholarship. To create sustainable change, long-term faculty development initiatives focused on mentorship are needed so that instructors can develop and implement sustainable curricula with local relevance. Experienced instructors seasoned in developing and implementing course-based undergraduate research experiences (CUREs) based on their own scholarship and local resources can convey their experiences to mentees interested in using these pedagogical techniques as the centerpiece of their own teaching. The Council on Undergraduate Research (CUR) Biology Division has created the Mentorship for Integrating Research Into the Classroom (MIRIC) program to provide a means for members with an interest in developing improved and sustainable active learning techniques to gain experience in this style of teaching through close, long-term interaction with a veteran teaching mentor. Developed from the former ASCB Mentorship in Active Learning and Teaching (MALT) program, MIRIC focuses on the development of instructors who wish to develop a dynamic CURE. Current and future life science instructors pair themselves up with seasoned veterans of CURE development and work with them and their students over the course of a semester or longer to develop a CURE that will allow the mentee to bring authentic research into his or her classes. In our pilot studies, we collected qualitative and quantitative data based on participant interviews and coding videos of student and instructor actions during classroom activity (Smith *et al*., 2013), respectively, that suggest that MIRIC mentorships have made positive gains in promoting sustainable active learning techniques among participants. Since then, we have surveyed the first cohort on both the success of their CURE development process as well as the benefits of the mentoring relationship on both the mentors and mentees using validated questions derived from Mathur (2012) and Pamuk and Thompson (2009).

P22/B22

Using modeling and animation to overcome previously-held misconceptions.

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Modeling of biological processes through drawing or animation is a skill that can be key to understanding essential concepts in biology. In fact, the development and construction of models is an essential practice for meeting the Next Generation Science Standards (www.nextgenscience.org) for K-12 science education and a key core competency in Vision and Change in Undergraduate Biology Education (www.visionandchange.org). From early life science classes in elementary and secondary school through graduate education drawing and animation are used effectively to cement key concepts. Unfortunately, early use of incorrect drawings and models can lead to misconceptions and a lack of competency in foundational areas that are often retained. To study ways to overcome misconceptions in biology, I have focused on the intentional construction of new models following observation of videos of an actual cellular process. One foundational cellular process that students frequently model incorrectly is mitosis. Incorrect models of mitosis lead to incorrect understanding of errors made in mitosis and more complex phenomena such as meiosis; two key topics in the upper-level Cytogenetics class offered at Bucknell University. In an effort to replace incorrect models retained from earlier study of mitosis, students started the semester by producing a short animation of mitosis, showing their baseline understanding of the process. Students then watched time-lapse videos of mitosis, carefully comparing

their own animations to the videos of the process in living cells. This comparison showed that students' models were often inaccurate. Students then reanimated mitosis based on their view of the process in living cells. A quiz given at the end of the semester (two months later) showed that students retained what they learned from re-modeling mitosis after watching the process in living cells. I propose that careful reflection on misconceptions and combining observation of processes in life with re-modeling those processes can help students overcome previously-held misconceptions.

P23/B23

Science Education Videos Improve Student Performance in Non-major and Intermediate Biology Laboratory Courses.

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Undergraduate biology laboratories emphasize hands-on skills. Descriptions of manual techniques required during laboratory instruction are often delivered via written instruction. We hypothesized that students who watch short, professionally produced high-quality instructional videos before performing a laboratory would achieve greater learning gains and self-efficacy than students whose pre-lab instruction was limited to handouts. We employed two videos in an intermediate molecular biology course at a small, liberal arts university and two videos in a non-majors biology course at a large, public research university. Both the videos and accompanying lab handout were assigned as a pre-lab assignment for 50% of the lab sections; the remaining 50% of the lab sections did not watch videos and only read the lab handout. A baseline survey assessment before the laboratory revealed no statistical difference between groups. Subsequently, both video and comparison groups were administered a pre- and post-lab exams. A post-lab self-efficacy survey was also administered to video groups. Our results reveal that in three out of the four laboratory classes, students who watched videos performed significantly better in both pre- and post-lab exams. For these students, we observed up to a two-fold increase in test scores on scientific concepts and techniques. For all four classes, 65-95% of the video group students reported that the video helped enhance their confidence, comprehension of concepts, and understanding of how to conduct the lab. We conclude that instructional videos can be used as an effective resource in enhancing existing curriculum of undergraduate science courses and significantly improving students' performance.

P24/B24

Making It Stick: the Story of CURE That Imparts Career Ready Skills.

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Researchers have used THP-1 cells to study the host response to implantable devices and biomaterials in *vitro*. Tissue contacting surfaces of implantable materials initiate a host inflammatory response characterized by many events, one of which includes macrophage attachment to the surface leading to degradation and failure of the material. Utilizing an established THP-1 adhesion assay and four-step pedagogical framework, an eight-week CURE was implemented which allows students to participate in the scientific process by testing scientifically viable substances which may prevent the host inflammatory response to implantable devices and biomaterials. Importantly, the host response to implantable materials is a large healthcare burden and strategies to prevent it are currently being investigated. This CURE was successfully developed, implemented, and assessed over two semesters

and is now routinely implemented in a private four-year university and a satellite campus of a public four-year university. We set out to evaluate our CURE to see if this experience enhanced not only students' acquisition of needed "hard" skills deemed important for future work in cell biology research, but several top soft skills considered essential for the nation's overall workforce. What we discovered sheds light not only into these areas, but the indisputable significance of CUREs as a checkpoint experience for student reflection on career choice and a potential window of opportunity for faculty mentorship and guidance on workforce preparedness and career options.

P25/B25

Student Perceptions of Iteration and Collaboration in Research during Laboratory Courses.

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Two important aspects of Course-based Research Experiences (CUREs) are the opportunities to collaborate and opportunities to iterate in the laboratory. Previous studies describe in-lab collaboration as an important facet of CUREs. We designed and ran CUREs or CURE modules at three different levels of an undergraduate Biology curriculum—Introductory, Intermediate, and Advanced—in order to provide research opportunities at a primarily undergraduate institution that afforded meaningful collaboration to students at different stages of their undergraduate careers. Introductory-level students who participated in a short CURE module reported that repetition is an important part of Biological research more often than their counterparts in a control course. At the intermediate level, students ran quality control genotyping on samples provided by a large research-oriented (R1) university within existing course content. At the end of the quarter, students had the opportunity to re-run an experiment of their choice, including the collaborative CURE experiment. Focus group interviews suggests that the CURE itself, and its focus on collaboration, was less salient to students than the opportunity to revisit experiments or protocols. Finally, advanced level students who participated in an *intra*mural CURE across disciplines were exposed to the multi-level analysis approach that is common in many published research studies. We used portions of the Laboratory Course Assessment Survey (LCAS) as well as open-ended questions to probe students' attitudes about collaboration and repetition in research projects, and found that repetition was, again, a salient part of their research experience. Through these examples, we hoped to determine best practices for introducing realistic scientific collaboration into CUREs. Our preliminary results suggest that collaboration may change how students strategize about problem solving. However, iteration and productive failure may be more immediately important to students' approaches to research projects.

P26/B26

Development and Implementation of a Cell Culture Module: Investigating the Molecular Mechanisms Behind the Survival and Proliferation of Metastatic Ovarian Carcinoma Cells.

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Course-based undergraduate research experiences (CUREs) provide research opportunities for students, and students engaged in a CURE course display increased engagement and understanding of the flow of biological research. Furthermore, this course model provides an authentic research experience that better prepares students for graduate programs and careers in STEM. Cell culture-based research projects are being implemented into undergraduate classrooms to increase understanding of cell

biology research and promote proficiency in scientific techniques utilized frequently across industrial and academic biomedical research. Here, a new and unique 15-week 400-level CURE Cellular Physiology Laboratory was designed with the main objectives being to promote understanding of the flow of biological research and promote mastery of cell culture techniques. In this course, the following work flow was used: 1. students are presented with primary literature about a biological problem, 2. students develop a biological question and plan experiments, 3. students complete experiments and analyze, and 4. students collaboratively write a final research paper and give a research presentation. In this course, students were presented with current research in epithelial ovarian carcinoma, a form of cancer that has a relatively low survival rate because it is often diagnosed after it has already progressed to stage III or stage IV. Thus, a better biological understanding of the disease could lead to improved treatments and higher survival rates. Through reading, students discover that in order for cancer cells to spread (or metastasize) to regional and distant sites, epithelial ovarian carcinoma cells must be able to overcome extracellular matrix (ECM)-detachment-induced effects, including combatting increased reactive oxygen species (ROS). In the Spring 2018 iteration, students worked collaboratively as a group to investigate the role of Peroxiredoxin 1 (PRDX1) in the survival and proliferation of ECM-detached ovarian carcinoma cells. Students learned and utilized cell culture techniques, including trypan blue exclusion assays, soft agar assays, and immunoblotting. After completion of experiments and analyses, students completed a final research paper and final research presentation. Students reported increased passion and understanding for cell biology. Observations suggest that students had an increased understanding of the flow of biological research and confidence in performing cell culture techniques by the end of the course. In conclusion, these results suggest that the objectives for the course were met. In the future, this course will be improved and enrollment expanded to provide more opportunities for cell culture-based research to students.

New Techniques in Light and Electron Microscopy

P27/B28

A Note on Cell Fixation Using Formaldehyde Or Paraformaldehyde.

M. Hammer, S. Flemming, D. Grunwald; UMASS Medical School, Worcester, MA.

A Note on Cell Fixation using Formaldehyde or Paraformaldehyde Mathias Hammer[1,2], Selene Flemming[1], David Grunwald[1] [1] RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA, USA [2] FB Biology, Technical University Darmstadt, Darmstadt, Germany E-MAIL: david.grunwald@umassmed.edu KEYWORDS: formaldehyde, smFISH, nucleus, membrane, microscopy, refractive index Cell fixations are nearly as old as microscopy. They have given us the ability to investigate the structures of cells, their compartments and to develop techniques like smFISH, which allow the study of intracellular interactions on a molecule level. These studies have resulted in the development of our understanding of life and its architecture. Over 20 years ago it was found that fixation with para-/formaldehyde influences the shape of the cell membrane**[1]**, still para-/formaldehyde is currently being used as fixative in many labeling protocols and the vast majority of FISH protocols. To investigate the effects that this type of fixation has on cell shape and subcellular structures, we used series dilutions of para-/formaldehyde on living cells and observed the 3D shape and changes in the refractive index of subcellular compartments in real-time using a new kind of microscopy technique**[2]**. We find that the cytoplamsic membrane builds up bubbles upon treatment with para-/formaldehyde.

These bubbles extend for x-y minutes and are easily removed during sub-sequent washing steps. In parallel subcellular structures show massive changes in their shape and refractive index, which includes changes to the shape of the nuclear membrane and the inner nuclear structure. We present a method that prevents these changes to the cell morphology by modifying the media composition through addition of iodixanol. [1] Brock R, Hamelers IH, Jovin TM. Comparison of fixation protocols for adherent cultured cells applied to a GFP fusion protein of the epidermal growth factor receptor. Cytometry. 1999 Apr 1;35(4):353-62. doi: 10.1002/(SICI)1097-0320(19990401)35:4<353::AID-CYTO8>3.0.CO;2-M. PubMed PMID: 10213201. [2] Cotte Y. Toy F, Jourdain, P, Pavillon N, Boss D, Magistretti P, Marquet P, Depeursinge C. Marker-free phase nanoscopy. Nature Photonics. 2013-02;7:113-117. doi: 10.1038/nphoton.2012.329.

P28/B29

Application of Advanced Microscopy Technology in Cell Biology and Disease Study.

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Recent advances in microscopy have led to the development of technology and instrumentation, capable of achieving higher resolution from centimeters to angstroms. However, optimal preservation of biological specimens remains a challenge. As a research driven microscopy core facility, our goal is to identify methods that offer superior preservation for a wide spectrum of samples through experimentation. These innovations, combined with the application of various advanced microscopy technologies, allow us to serve the interests of researchers from multiple disciplines, from developmental biology to disease study. T cells are specialized lymphocytes that play a central role in cell-mediated immunity, including antiviral immunity. Studying morphological changes in mitochondria of T cells in cancer mouse models, is an important aspect of cancer research. However, preserving tiny amounts of flow-sorted T cells from mice for ultrastructural study, is technical challenging. We use a modified protocol, reduced osmium-thiocarbohydrzide-osmium (rOTO), with additional uranyl acetate and lead aspartate staining, to further increase heavy-metal content, such that the membrane structure of T cell mitochondria is greatly enhanced. This *en bloc* staining method can also provide high contrast, increase sample conductivity, and reduces charging issues during serial block face scanning electron microscopy (SBF-SEM) imaging. A similar sample processing approach was used to study host cellular compartments that serve as *Wolbachia* niches. The rOTO protocol produces a clear outline of *Wolbachia*, which makes it much easier to distinguish the membrane of *Wolbachia* from other cytoplasmic organelles in both cultured JW18 cells and the ovary of *Drosophila*. SBF-SEM results provided strong evidence that *Wolbachia* reside intracellularly, within modified host endoplasmic reticulum niches. Arrhythmogenic cardiomyopathy (AC; also known as ‘arrhythmogenic right ventricular cardiomyopathy’ or ‘ARVC’) is an important cause of sudden cardiac death in young people. AC is a genetic cardiomyopathy where most of the disease-causing mutations occur in proteins that form part of the desmosome, such as plakophilin-2 (PKP2). The relationship between PKP2 and connexin 43 (Cx43), a primary component of gap junctions, is key to the pathogenesis of AC. By combining single molecule imaging technology, correlative light and electron microscopy and three-dimensional electron microscopy, we demonstrated that the outer edge of Cx43 crosstalk with PKP2 clusters, and the dimension of these clusters depend on an kyrin G expression. We also demonstrated that $Na_v1.5$ molecules are major contributors to cardiac sodium current, and loss of $Na_v1.5$ expression reduces intercellular adhesion strength.

P29/B30

Scale-free Tracking Microscopy of Freely Suspended Cells: Towards Connecting Cell Biology and Ocean Ecology.

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Many cells spend their entire lives freely-suspended in a fluid, rarely encountering any substrate. Uni-cellular marine plankton epitomize this lifestyle, where single cells can travel several tens to hundreds of meters vertically in the water column, as part of the largest daily bio-mass migration on our planet. Such migrations are key components of vertical material fluxes in the ocean and drive planetary-scale biogeochemical cycles, and form a major component of oceanic carbon sequestration. Measuring sub-cellular-scale processes in single cells while allowing free movement over ecological-scale distances is, therefore, crucial for mechanistically understanding the cell biology and ecology of the living ocean. However imaging over large distances along the axis of gravity is a considerable challenge for conventional microscopy: Given the tradeoff between optical resolution and field-of-view, how can one measure single organisms or cells, at microscale resolution, while allowing them to freely move hundreds of meters in the vertical direction? Here we present a solution in the form of a scale-free, vertical tracking microscope, based on a circular “hydrodynamic-treadmill” for single cells, with no bounds for motion along the axis of gravity. Our method demonstrates a new paradigm for simultaneous multi-scale measurement where microscale characteristics like cell-state and organismal behavior can be directly connected to macroscale outcomes like depth in the water column. Using our tool, we demonstrate novel biophysical measurements in two classes of ecologically relevant plankton. In single-celled diatoms spanning four species, we discovered rapid density fluctuations over millisecond time-scales by concurrently measuring cell behavior (vertical sinking speed) and cell’s molecular state by synthesizing our tracking microscopy method with fluorescence imaging of signaling reporters. In the dinoflagellate cells *P. noctiluca*, we observed, for the first time, cell-division in suspension, far from substrates and resolve microscale fluctuations in cell density during division and rapid changes in cell volume post-division. Finally, we combined our tracking methodology with environmental patterning of light, chemical species and ambient pressure to bring the virtual-reality paradigm, which is well-established in neuroscience, to cell biology in the ocean. We anticipate that our method will open up new avenues for understanding key biophysical processes in our oceans at sub-cellular resolution, and allow connections between cell biology and ecology. Beyond the oceans, we foresee that our method will allow novel cell-biological measurements by freeing cells from the confines of the coverslip.

P30/B31

Local Surface Plasmon Resonance of Gold Nanoparticles as a Correlative Light and Electron Microscopy Tag.

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Correlative light and electron microscopy (CLEM) is powerful method to reveal the relationship between nano structures and functions in cells. We previously reported the CLEM of optical microscopy (OM) and transmission electron microscopy (TEM) using a silicon nitride (SiN) film. The same sample mounted on the SiN film could be observed seamlessly by OM and TEM using an exchangeable retainer. However, this method had some limitations for applications to biological samples. For example, more stable

fixation of samples was needed for TEM using strong fixatives (glutaraldehyde and osmium tetroxide), which usually reduced or quenched the fluorescence of fluorescent proteins or dyes. Furthermore, thin sectioning of the samples was required to transmit the electron beam (about 100 nm less). As a result, these thin samples contained little fluorescent substances, resulting in very low image contrast of OM. In other words, a new tag for OM, which is resistant to crosslinking by aldehyde and oxidation by osmium tetroxide and possesses strong luminescence properties from small volumes, is required to apply CLEM to biological samples. In this study, we investigated application of localized surface plasmon resonance (LSPR) of metal nanoparticles (NPs) to the CLEM tag for biological samples. The optical properties of metal NPs are determined by the interaction of electrons and light near their surface. When metal NPs are irradiated with light, collective oscillation of electrons near the surface of metal NPs occur. This collective oscillation of electrons by light is LSPR. LSPR causes strong absorption and scattering light at specific wavelengths. This wavelength strongly depends on the kind, size, shape, surface modification, and aggregation state of metal NPs. As results of investigations, Gold NPs in ultra-thin sections for TEM revealed that LSPR could be observed by optical microscopy when those sizes of 20 nm or greater. Gold NPs at sizes less than 20 nm could be observed using the gold enhancement method. Therefore, this CLEM tag could be applied to immunoelectron microscopy using this gold enhancement method. These results suggested that gold NPs were useful as a CLEM tag for biological samples.

P31/B32

An Elemental Approach to Semi-automated Segmentation of Organelles in Three-dimensional Scanning Electron Microscopy Enables Rapid an alysis of Cell Ultrastructure.

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Three-dimensional (3D) scanning electron microscopy (SEM) techniques such as serial block face scanning electron microscopy (SBFSEM), focused ion beam scanning electron microscopy (FIBSEM) and array tomography produce increasingly large datasets with an immense amount of information. The applications for 3D SEM have broadened beyond the neurobiology and connectomics fields to other areas of biology, including cell biology in a diverse range of species (Borrett and Hughes, 2016). An alysing these datasets is a huge undertaking, with the major resource bottleneck being researcher time (Peddie and Collinson, 2014). Selective staining techniques can aid in rapid analysis by enabling researchers to threshold data based on stain intensity (Kittelmann *et al*., 2016), but there are a limited range of stains that can specifically target regions or organelles of interest and the technique is hampered by the inclusion of artefacts at similar greyscale thresholds. The aim of this investigation was to test if elemental analysis using energy dispersive X-ray spectrometry (EDS) could be used to identify endogenous elements and stains in biological samples and separate them from common artefacts. A model system of liver tissue was used to examine if elemental mapping could be used in combination with array tomography to generate element-based masks for semi-automated segmentation of image data. Liver tissue was prepared using standard fixation and staining techniques for transmission electron microscopy. Serial sections were cut at a thickness of 100nm and collected onto carbon coated Kapton tape. Array tomography was used to collect BSE images and EDS maps of whole hepatocytes. EDS maps were used to produce 3D masks, differentiating between lipid droplets and similar greyscale structure within cells. The masks were applied to 3D array tomography datasets, enabling volumetric and quantitative analysis in a shorter amount of time than the equivalent manual segmentation. EDS in combination with 3D SEM techniques can be used to produce element-based masks rapid and semi-

automated segmentation of backscattered electron (BSE) image data. Borrett, S. An d Hughes, L., 2016. Reporting methods for processing and analysis of data from serial block face scanning electron microscopy. *Journal of microscopy*, *263*(1), pp.3-9. Peddie, C.J. An d Collinson, L.M., 2014. Exploring the third dimension: volume electron microscopy comes of age. *Micron*, *61*, pp.9-19. Kittelmann, M., Hawes, C. An d Hughes, L., 2016. Serial block face scanning electron microscopy and the reconstruction of plant cell membrane systems. *Journal of microscopy*, *263*(2), pp.200-211.

P32/B33

CryoAPEX Paves the Way for the Discovery of an Unconventional Membrane Transport Pathway Induced by Alphaviruses.

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The determination of specific localization of proteins in *situ* at a high resolution is the first step towards comprehensive understanding of form and function within the subcellular landscape. However, available electron microscopy methods exhibit inadequate ultrastructural preservation leading to incomplete and artifactual localization data from attempted 3D reconstructions. Our recently published method, CryoAPEX exhibits excellent ultrastructural preservation and enables localization of a protein in 3D via EM tomography by coupling peroxidase (APEX2) tagging and cryofixation of cells using high pressure freezing. The method thus closes the gap in understanding the critical processes within cells during endogenous or pathogenic events that results in the morpho-functional remodeling of membranes and organelles. The work depicted here exhibits the application of CryoAPEX to dissect the trafficking of envelope glycoprotein (E2) of an alphavirus, a plus-strand RNA arbovirus. Although, a well-studied virus, the details of the membrane trafficking of the alphavirus E2 in its mammalian and mosquito hosts remains unclear. Here we morphologically dissect the trafficking pathway of E2 in mammalian and mosquito cells by constructing a virus with the APEX2-peroxidase gene inserted in-frame within the E2 gene. This study reveals that the virus remodels the Golgi apparatus and engineers an unconventional membrane transport system for the trafficking of E2 from the Golgi to the plasma membrane, the site of its egress. We identify mutations that results in abrogation of a morphological subclass within these pleomorphic transport vesicles resulting in a sharp drop in virus egress. Thus we show that though these vesicles originates from the same organelle they are born out of distinct mechanisms. We further show that these transport intermediate undergoes a morphological flux as the infection progresses resulting in complete remodeling of the Golgi apparatus. We also used APEX2-tagged organelle markers in parallel to ascertain the origin of these pleomorphic transport intermediates at various stages of infection. In summary, this work showcases a powerful application of CryoAPEX in the discovery of a pathogen induced unconventional trafficking pathway and paves the way for such future discoveries in disease and microbial pathogenesis.

P33/B34

Cryo-em Structure of the Stressosome Complex.

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the stressosome complex is a virus-like capsid macromolecules that is activated in response to diverse environmental stresses including heat, ethanol and osmotic shock. It is known that the macromolecule is directly involved in the upregulation of Sig*B*-dependent transcription pathway. Previous studies demonstrated intramolecular self-assembly within the complex under symmetry reconstruction of cryo-EM, however the structural details in assembly of stressosome is still unclear. Here, we present reliable assembly model of the RsbRA/RsbS stressosome complex based on the crystal structure of RsbS icosahedron with cryo-EM structure under symmetry and symmetry-free reconstructions determined at 4.1, 7.1 and 9.1 Å, respectively. The crystal structure formed 60 monomers for RsbS fitted well into the electron densities of icosahedral cryo-EM model. Furthermore, the RsbRA dimers of 22 protrusions were also observed in the envelope of symmetry-free reconstruction map. Following these suggestive results, our study can aid further structural investigation of various types of stressosome complexes and answer unsolved questions about the stressosome in the coming future.

P34/B35

Multi-patterned Phase Target to Benchmark Phase Contrast-based Microscopies of Cell Cultures.

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Phase contrast microscopy is routinely used to monitor cell cultures in academic, commercial and government laboratories. Because it is a non-destructive imaging technique, phase contrast evaluations of cell cultures can be used to make critical decisions about whether to harvest cells or to change growth conditions. A challenge with conventional phase microscopy is that cellular evaluations are qualitative and can be difficult to reproduce due to the complexities of the optical train in the microscope setup. For commercial applications of cells, such as in therapies and biotechnology, quantitative and non-invasive strategies to evaluate cell culture are required. Quantitative phase imaging (QPI) is an emerging imaging modality that can address this gap in phase contrast microscopy. QPI enables quantitative measurement of the optical pathlength difference created as light passes cells. There is a lack of reference materials and protocols to enable comparability and assure reproducibility of the imaging results. To ensure confidence in the QPI techniques, we present a quad-patterned glass etched glass target with optical features (sizes and phase shifts similar to mammalian cells) as a prototype physical standard to benchmark the performance of phase contrast microscopy and QPI. The quad-pattern target contains 1) a featureless flat region of glass (< 2 nm roughness), 2) a lateral grating with 40 µm period and 500 nm step height, 3) a square grid of cylindrical features with discrete optical volumes (150 μm^3), and 4) a Siemens star resolution target with measured spatial resolution down to 360 nm. We have characterized both the physical height and optical pathlength difference of the optical features, and the spatial resolution of the quad-patterned material with orthogonal measurement systems. We present cell imaging examples for both phase contrast microscopy and QPI that show how to use our reference material to rectify a misaligned phase ring for phase contrast or an incorrectly set condenser aperture for QPI. This type of material may be useful for the emerging growth area of label-free nondestructive live-cell imaging and could serve the measurement assurance needs for quantitative cell biology in the engineering biology and healthcare fields.

P35/B36

Universal EM Connectomic an alyses by Deep Learning Powered App-matching Image Conversion.

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Deep learning (DL) is emerging as a powerful tool that has attracted a lot of interest in microscopy image analysis. Using deep learning for automated 3D EM image boundary detection is a promising new development. However, this technology is currently only used by a small number of pioneering research groups. This is largely due to four major practical hurdles: (1) the requirement for multiple highly specialized and disjointed software libraries or tools to cover the entire DL train-apply workflow; (2) the need to have extensive expertise to fine-tune the training process; (3) the need to access high-performance hardware to train DL networks, and (4) the difficulty and time-consuming process of ground truth (GT) creation. Furthermore, the resulting deep models are specific to the trained experimental and imaging conditions (called “domain”) and are not readily applicable to images from other domains without significant re-training. We are developing a novel deep learning powered app-matching image conversion framework that converts images from a new domain to mimic the images from the domain where an application model (called “App”) is created and validated. Therefore, the validated App from a training domain can be applied to a new domain by converting new domain images to the training domain. This renders the App universally applicable without new domain specific re-training. We validated the app-matching image conversion framework in the 3D EM image boundary detection applications. A U-net (the App) is trained to segment 3D neurites in EM images from the ISBI 2013 challenge (the training domain). The App is applied to new EM images acquired at the Rachel Wong Lab from the University of Washington (new domain). The new domain images are converted through app-matching image conversion and then the App is applied. The results are close to a new U-net specifically trained using the new domain data. Furthermore, we demonstrated that the app-matching image converter can be trained with as few as a single image from the new domain. This represents a significant advantage in practical applications. We are working on application enhanced conversion and artifact rejection that should further improve the applicability and performance of our novel app-matching image conversion framework.

P36/B37

***In Situ* Measurement of Protein and Lipid Mass by Normalized Raman Imaging.**

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Cell growth is the basis for myriad biological processes ranging from development, regeneration and tumor growth. Diverse extracellular signals such as growth factors, hormones, nutrients, and cell-to-cell contact are transduced by the signaling pathway to regulate growth. Yet it is still an open question how the integration of diverse inputs results in cell growth or maintains cells at the right size in the face of massive protein turnover. Accurate measurement of cell size is critical for probing these questions. State-of-art techniques such as quantitative phase microscopy, suspended microchannel resonator, and novel fluorescence reporter made it possible to measure cellular dry mass in higher precision and throughput than ever. Nevertheless, the existing methods require suspended cells or in vitro cell

cultures by which the contextual information of the tissue is lost. While the cell volume can be measured by confocal microscopy, there is no existing method to measure cellular dry mass or protein mass in *situ*. Here we present Normalized Raman Imaging (NoRI) that fills the technology gap by providing the absolute concentrations of total protein, total lipid, and water of cells and subcellular compartments in *situ*. NoRI achieves the absolute quantification by combining Stimulated Raman scattering (SRS) microscopy with a novel computational algorithm that removes the effect of light scattering in thick tissue samples. NoRI is a label-free technique that can measure live or fixed tissue sections, cultured cells and small model organisms without any use of staining. The single cell protein mass and lipid mass can be acquired by integrating the respective concentrations over the cell volume. NoRI reveals that cell types and tissue types have characteristic protein and lipid density, which may change with developmental or disease processes, and opens a door to many interesting questions about the regulation of cell growth in tissue context.

P37/B38

Optimization of Quantitative Phase Microscopy Reveals Autonomous Growth Rate Oscillation in Mammalian Cells.

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Growth is a fundamental property of cell physiology. However, the understanding of its regulation in mammalian cells has been thwarted by the difficulties in growth rate measurement at the single-cell level. Here we report an optimized Quantitative Phase Microscopy (QPM) for longitudinal and large scale measurement of cell growth in adherent cells by improved image processing algorithms and automated cell tracking software. The measurement accuracy is improved by more than two folds. Using the new system, we reveal a remarkable growth rate oscillation throughout cell cycle in several different cell lines, which is independent of cell cycle progression but coupled to cell division. The growth rate oscillation may be a general feature of mammalian cell growth regulation.

P38/B39

Correlative Transformation and Visualization Tool for Correlative Light-electron Microscopy (clem) analysis.

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Recent technological advancements in microscopy techniques, labeling methods, preparation procedure, data analysis informatics and computing infrastructures have made high-resolution imaging and visualization of large tissue blocks a possibility. Armed by the promising advancements in elucidating and quantifying the connectomics of brain networks with subcellular resolution using electron microscopy (EM). There are increased interests in hybrid approaches correlating functional fluorescence microscopy data and ultrastructural information from EM in a common biological context, correlative light electron microscopy (CLEM). We are developing a correlative transformation and visualization tool to perform automated image registration for CLEM analysis. General purpose landmark and intensity-based image registration approaches were implemented to register a LM image with a corresponding EM image. Both rigid registration and non-rigid registration methods were developed and applied to the CLEM dataset for comparisons. The 3D image viewer supports correlative

visualization of 3D LM and EM volumes in a single window. The functionality of clipping/orthographic planes enables viewing of the volume cross sections in the X, Y, and Z directions, or in any arbitrary orientations. This feature becomes very useful when certain objects could only be observed or found in a certain orientation. We validated the correlative transformation and visualization tool using a retina CLEM dataset acquired at the Wong Lab. The tool is integrated with the 3D visualization environment in Aivia for fast viewing of registered LM and EM volumes. This greatly speeds up the studies for neuron circuitry identification and novel cell type discoveries. We will continue to improve the correlative transformation and visualization tool in an Aivia prototype by introducing more 3D visualization options and adding support for measurements and data structures for correlative studies.

New Techniques in Cell Biology 1

P39/B40

Modeling the Relationship between Cell Size and Division Number in Chlamydomonas Using a Stochastic Hybrid System Approach.

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Active maintenance of cell size through homeostatic mechanisms is thought to promote fitness in proliferating cells. Size homeostasis in green alga Chlamydomonas is under the control of both “Sizer” and “Timer” mechanisms that govern its multiple fission cell cycle. In multiple fission cells alternate between a prolonged G1 phase during which daughters may enlarge many-fold in size, and a division phase with rapid alternating rounds of DNA synthesis and mitosis (S/M phase). The “Sizer” governs the minimum size a cell must reach in order to transition into S/M phase, and it determines how many rounds of S/M a mother cell will undergo such that daughter size distributions are uniform. The “Timer” enforces a five to eight hour delay between reaching the minimum size for division and entering S/M phase. The ability to control the extent of growth in G1 phase using duration/intensity of available light in phototrophic cultures enables production of different sized mother cell populations. It is well established that larger mother cells divide more times on average than smaller mother cells so that daughter populations are uniform regardless of the size of the mother cell population that produced them. While, on a population level, cell division behavior is reproducible, on a single cell level there may be stochastic variability whose origins and contributions to size homeostasis are not well understood. We established a microscopy-based method to track the relationship between cell size and division number in individual cells. We found that while in general, larger mother cells divide more times than smaller mother cells, individual cells of the same size do not always divide the same number of times. Reciprocally, when grouped by cell division number, mother cells that produced a given number of daughters (e.g., 2, 4, 8, and 16) occupied a range of sizes that often exceeded the two-fold range which would be expected if the mitotic sizer mechanism operated perfectly. We are using modeling and simulation approaches to investigate how stochastic behaviors at the single cell level in Chlamydomonas can lead to predictable and uniform behavior at the population level. Empirical statistical properties of the relationship between cell size and division number are fit into the probability models. Stochastic hybrid models can be used for these simulations and will help determine whether the same set of

stochastic parameters rules govern size control in haploid versus diploid cells or in mutants that are defective in cell size control.

P40/B41

Manipulation of Cellular Rna Using an Artificial Rna Binding Protein.

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Engineering of protein that target specific DNA sequence is expected as a useful tool for genome editing technology. Although genome-editing technologies have been established, the protein engineering that targets a particular RNA is still infancy. The PPR (pentatricopeptide repeat) motif-containing protein is a sequence-specific RNA binding protein family, and involved in multiple aspects of organelle RNA metabolisms. PPR proteins consist of a tandem array of PPR motifs (degenerated 35 amino acids) in variety repeat length (2-27 repeats). We solved the PPR-RNA recognition mechanisms: one PPR motif corresponds to one nucleotide, and the RNA nucleotide specificity is determined by the combination of amino acids at three particular positions within a motif. Using the knowledge of mechanism of PPR and RNA binding, we are able to create the various artificial sequence-specific RNA binding protein. Here we show the binding performance of our artificial PPR protein and its application to manipulate cellular RNA. First of all, we developed the efficiently PPR cloning systems, and could construct PPRs that contain various number of PPR repeats. Designed PPR proteins for 23 kinds of target sequences were constructed and analyzed their binding affinity and specificity. The results of binding affinity of all combination among PPRs and RNAs indicated that a sequence-specific RNA binding protein could be created with a construction probability of 80%. Using this artificial RNA binding protein, we developed a cellular RNA manipulation tool that control alternative splicing. In order to test whether designed PPR protein can control splicing through the binding to target RNA sequence of pre-mRNA in the cell, target sequences were selected from exon or intron of the splicing reporter, and their PPRs were constructed. Co-transfection of splicing reporter and PPR expression plasmid in HEK293T cell showed that target-specific binding artificial PPR protein can change the skipping ratio of target exon. Moreover, this PPR tools could be alter the amount of splicing variants of an endogenous RNA in the mammalian cell. Thus, our PPR technology would be useful to manipulates the splicing in the cell.

P41/B42

Immuno-SABER Enables Highly Multiplexed and Amplified Protein Imaging in Tissues.

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Mapping the molecular composition of individual cells in their native environment is critical to understand the cellular and subcellular organization of tissues in healthy and diseased states. However, conventional fluorescence imaging methods offer limited (typically <5) multiplexing due to spectral overlap. Sensitivity is the other limiting factor owing to high autofluorescence and scattering in the tissue samples, making it difficult to visualize low abundance targets. To achieve the desired high sensitivity and throughput, a clear need exists for a robust and scalable signal amplification method compatible with rapid multiplexing. To address this challenge, we developed a new in *situ* protein imaging method, **Immunostaining with Signal Amplification by Exchange Reaction (Immuno-SABER)**[1]. To simultaneously achieve high multiplexing and sensitivity, SABER[1,2] leverages: **1)** Multiplexing via DNA-

Exchange-Imaging (DEI) that enables fast and spectrally-unlimited imaging through single-step immunostaining with DNA-barcoded antibodies[3]. **2)** a new in *situ* signal amplification method, based on Primer Exchange Reaction, can autonomously synthesize long single-stranded DNA concatemers from short primer sequences using a novel catalytic DNA hairpin structure in a pre-programmed fashion[4]. SABER offers independently programmable signal amplification without in *situ* enzymatic reactions, and intrinsic scalability to rapidly amplify and visualize a large number of targets. We validated SABER for imaging of proteins in diverse sample types including cells, cryo and FFPE sections, and whole mounts. We demonstrated tunable 5 to 180-fold amplification, covering the full signal range conventionally achieved by secondary antibodies to tyramide signal amplification, as well as simultaneous signal amplification for 10 different proteins using standard equipment and workflows. We further combined SABER with Expansion Microscopy to enable rapidly multiplexed super-resolution tissue imaging. Immuno-SABER presents an effective and accessible platform for multiplexed imaging of proteins with high sensitivity and throughput, and is applicable for a broad range of applications including molecular atlases, biomarker discovery, and digital pathology. [1] Saka S.K. et al. Highly multiplexed in situ protein imaging with signal amplification by Immuno-SABER. Nat Biotechnol*. In press.* [2] Kishi J.Y. et al. SABER amplifies FISH: enhanced multiplexed imaging of RNA and DNA in cells and tissues. Nat Methods 16, 533-544 (2019). [3] Wang Y. et al. Rapid Sequential in situ Multiplexing with DNA Exchange Imaging in Neuronal Cells and Tissues. Nano Lett 17, 6131-6139 (2017). [4] Kishi J.Y. et al. Programmable autonomous synthesis of single-stranded DNA. Nat Chem 10, 155-164 (2018).

P42/B43

Granger-causality Network Inference Identifies Functional Relations among Redundant Actin Modulators in Lamellipodia.

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Crawling cells assemble and disassemble filamentous actin mesh at the leading edge within as short as a minute in order to generate protrusive forces. This process is regulated by numerous actin modulators with substantial functional overlap and entanglement. Understanding the relations between these regulators has been challenging. Conventional molecular perturbation tends to fail as the redundancy between regulators results in immediate compensation. Moreover, the process is highly dynamic and localized in space, which results in an apparent heterogeneity of regulatory states. Here we propose a statistical causal inference framework that builds on established computer vision for morphodynamic profiling to determine directed cause-effect relations between the activities of actin modulators. Our method is based on the notion that signals of causative activity possess indispensable information in explaining the signals of an effector activity, and it extends this concept of Granger-causality inference to the massive spatially and temporally resolved data that can be acquired from live cell imaging. The framework automatically generates Granger-causality network diagrams between edge motion and observed modulator activity. Application of this mathematical framework to the combinatorial imaging of actin, Arp2/3 and VASP demonstrates, as expected, that actin assembly causes edge motion only in a narrow zone from the cell edge in U2OS osteosarcoma cells. Interestingly, actin assembly out of this narrow zone, while positively correlating with edge motion, does not have causal effect. This shows that our inference properly distinguishes causation from correlation. We then find that Arp2/3 recruitment has a strong causal effect on edge motion, mediated by actin assembly. This causal chain applies only within the same narrow zone from the edge, whereas causal links from Arp2/3 to actin more distant to the cell edge have no effect on edge motion. We also find a flow of feedback relations from actin to

Arp2/3, confirming the long-standing notion that a dendritic network architecture yields a mechanism for self-supporting, exponential growth. Applying this approach to VASP and Arp2/3 imaging, the causal network diagram reveals that these two actin regulators have Granger-causal effects on edge motion, yet operate independently. These are the first examples of a novel approach to probing the functional relations in molecular systems with strongly redundant components. We are now prepared to apply this framework to get deeper insight of the mechanisms of actin regulation in lamellipodia and to other systems of similar complexity.

P43/B44

Targeted and Selective Degradation of Mitotic Kinase AURKA by a New PROTAC Tool.

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Targeted protein degradation tools are becoming a new therapeutic modality, allowing small molecule ligands to be reformulated as bifunctional molecules (so-called 'PROTACs') that recruit a ubiquitin ligase to the target of interest, leading to ubiquitination of the target and its destruction via the ubiquitin-proteasome system. This new paradigm of 'event-driven' rather than occupancy-based pharmacology holds great hope for the development of catalytic drugs able to work at lower doses and with higher specificity than the ligands from which they are derived, and with improved pharmacodynamics. Here we describe the functional characterization of a small molecule PROTAC derived from AURKA inhibitor MLN8237 (Alisertib). We describe the efficient and remarkably specific destruction of both endogenous and overexpressed AURKA elicited by PROTAC treatment, and explore the phenotypic consequences of these events.

P44/B45

Automated Passaging, Feeding and Plating on Glass of Gene-edited Human Inducible Pluripotent Stem Cvells for High-throughput 3d Live Cell Microscopy Pipeline.

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The Allen Institute for Cell Science combines genomics, gene editing, and 3D live cell imaging of cell organization to identify and define the various normal and pathological states in which stem cells reside and to unlock the mechanisms by which cells transition among these states. We do this using a collection of human induced pluripotent stem cell (hiPSC) lines expressing green fluorescent protein tags on proteins identifying specific cellular organelles and structures (**the Allen Cell Collection at allencell.org**). To produce large numbers of standardized images, we developed an automated hiPSC culture procedure. We use the Hamilton Robotics Microlab Star platform to generate imaging plates with uniform and reproducible cellular confluency and morphology. Here we provide specific parameters, such as the movements of the plates across the deck, the angle and speed of the aspiration and dispensing of media, the seeding strategies and the timing of every step that we optimized. This approach was developed and applied to coating, seeding, passaging and feeding procedures for cell expansion in 6-well plastic plates, and 96-well glass bottom imaging plates. The overall optimization procedure aimed to preserve the undifferentiated state, maintain pluripotency, and prevent karyotyping abnormality of our gene-edited hiPSC lines for up to 10 passages. We present a side by side comparison of quality control results obtained from manual and automated operations. We also developed an automated image-based colony segmentation pipeline to measure and track colony growth

characteristics over time. The colony feature measurements (number, size, textures, etc.) are used to rank 96-well plates and ensure consistent and adequate quality control. We have also implemented cell passaging based on image-based confluency calculations to eliminate the need for manual cell counting steps. In addition to providing higher uniformity, reproducibility, and overall cell quality for imaging samples, the standardized automation protocol ensures consistency by removing operator to operator variability and potential error or bias introduced by manual repetitive tasks and fatigue of the operators.

P45/B46

Optogenetic Activation of Intracellular Antibodies for Direct Modulation of Endogenous Proteins.

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Intracellular antibodies, such as nanobodies and single-chain variable fragments (scFv), have become powerful tools for imaging, modulating, and neutralizing endogenous target proteins. Although a variety of antibody engineering techniques have been developed, an optogenetic tool for activating intracellular antibody to precisely control its target protein has not been reported. Here, we describe an optogenetically activatable intracellular antibody (Optobody) consisting of split antibody fragments and blue light-mediated heterodimerization domains. Blue light stimulation activates the optobody, inducing it to capture its target protein and subsequently inhibit the target. We expanded this optobody platform by generating various optobodies from previously developed intracellular antibodies, and demonstrated that photoactivation of a gelsolin (GSN) optobody and β2 adrenergic receptor (β2AR) optobody shut down endogenous GSN activity and β2AR signaling, respectively. Applying our novel optogenetic platform to the broad pool of available intracellular antibodies will facilitate optogenetic manipulation of various endogenous proteins and may provide a basis for designing potential inducible drugs.

P46/B47

Measuring T Cell Avidity and Enrichment Using Acoustic Force-based Technology.

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The key driver for effective immune cell therapies is the overall binding strength of the immune cell and the target cell (e.g. tumor cells). The overall strength is known as ‘avidity’, a parameter reflecting interaction efficiency. The key to success for immune cell therapies is generating effective and long-lasting immune responses. The avidity of an immune cell to its target is predicative of its functional potency, but current techniques to measure avidity are low-throughput and ineffective. Herein, we describe the use of acoustic forces to discriminate immune cells based on their avidity to tumor cells. The force required to separate a cell from its target is called the ‘rupture force’, and in this study, we were able to identify the rupture forces of tumor-specific and non-specific T cells and enrich these different populations for downstream characterization. T cells from a healthy donor were transduced with either a non-relevant or a melanoma recognizing T cell receptor and selected with puromycin. Melanoma cells were seeded in the flow cell and allowed to adhere overnight to form a monolayer. For confocal experiments, CFSE and Cell Trace far red stained T cells were mixed in a 1:1 ratio before co-culturing them in the flow cell. T cells engineered with a melanoma antigen-recognizing T-cell receptor needed 6 times more force than non-specific T cells to be separated from the melanoma target cells. Furthermore, 1.4 to 3.6-fold enrichment of high-avidity T cells was obtained from a mixed population of specific and non-specific T cells using acoustic forces. These findings indicate that melanoma-specific T

cells bind with a higher avidity than non-specific T cells and that they can be separated with this approach. In conclusion, we demonstrate a novel method to measure cell avidity and sort cells by utilizing acoustic forces. In addition, this technology is applicable to other research fields. The avidity of different CAR T cell clones to their respective target cells has been measured, serving as an in vitro predictor of CAR T cell functional potency.

P47/B48

OpenCell: Systematic Profiling of Intracellular Organization in Space and Time.

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Uncovering the principles driving intracellular organization requires a multi-disciplinary description of the internal architecture of cells. Where are all proteins in a cell localized? Can we map the full network of protein-protein interactions? How can we capture dynamic changes occurring - for example during cell division or differentiation? To address these questions, scalable methods are needed to quantitatively and systematically measure cellular organization in space and time. Here, we present the latest generation of tools we have developed for this undertaking. First, by using the split mNeonGreen system in human embryonic kidney cells, we have expanded robust CRISPR-based methods for the rapid generation of over 1,000 endogenously tagged fluorescent human cell lines. This includes automating the design and analysis of CRISPR editing experiments. Second, we have established optimized protocols for the high-throughput generation of live-cell 3D imaging and interaction proteomics data. Finally, we have implemented open-source automation solutions to streamline tissue culture and clonal selection. These innovations form the basis of OpenCell, our collection of live-cell fluorescence imaging and proteomics data for the genome-wide description of protein function and cellular architecture.

P48/B49

Dynamics and Functions of Antifreeze Proteins in Transgenic *Caenorhabditis Elegans* at Freezing Environments.

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Antifreeze proteins (AFPs) bind to water molecules organized in ice crystals and modify their shape. The crystal structures of several AFPs have been determined by X-ray crystallography analyses, and the surface complementarity between AFP and water on an ice plane have been evaluated by many structural docking studies. However, less is known regarding whether the ice-binding ability of AFPs actually work in *vivo*, and improve the freezing tolerance in living organisms. To demonstrate this, we generated a transgenic lineage of the nematode *Caenorhabditis elegans* that expresses AFP, and first observed the survival rate of *C. elegans* after freezing exposure. Interestingly, the wild-type AFP improved the survival rate of AFP-expressing *C. elegans* at freezing temperatures (-5°C), whereas the

AFP mutant exhibiting low ice-binding ability did not improve the survival rate at -5°C exposure. These results indicate that ice-binding property is a key factor responsible for freezing tolerance in *C. elegans*. We further observed the AFP dynamical motion in *vivo* for the ice-binding ability by X-ray single molecular observation. Diffracted X-ray Blinking (DXB) evaluates the single molecular motion using the time-resolved X-ray diffraction images from gold-nano crystals. Gold-nano crystals as motion probe were attached AFP of the intestinal cells in *C. elegans*. The motion of wild-type AFP in *vivo* gradually decreased, whereas that of the AFP mutant transiently increased, when the temperature was decreased under 0°C. These results reasonably agree with simulation of AFP molecular dynamics, suggesting that it is important to bind stably AFP to ice-crystals for the tolerance in the living animal. Furthermore, X-ray diffraction from ice crystals were observed during -20°C exposure, and showed its motion dependent on AFP motion.

P49/B50

Towards "Autonomous" Microscopy: Artificial Intelligence Microscopy Screening (aims).

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In cell biology, the identification of new pathways via genetic screens has been a central discovery strategy. Historically, this work was performed most readily in bacteria and yeast; however, it is important to advance this platform in mammalian cells. RNAi-based screens have been used to elucidate pathways in human cells. This approach has relied on two major strategies: arrayed screens, which have high specificity but require the production of each RNAi separately creating a technical bottleneck, and pooled screens, in which production is easier, but specificity and reproducibility suffer. To overcome these limitations, we have developed a novel screening approach termed **A**rtificial-**I**ntelligence **M**icroscopy **S**creening (designated AIMS). The new platform "converts" single cells into "separate" wells by applying machine learning and deep learning algorithms to detect subcellular phenotypes. In brief, a genome-wide suppressor screen is performed on cells expressing dCas9 (CRISPR inhibition) by infecting a single well of cells with pooled guide-RNA (gRNA)-expressing lentivirus so that every cell will express a distinct gRNA. These cells also stably-express a photo-activatable red fluorescent protein (pa-mCherry). Before the screen, a deep learning neural network model is trained on several examples of the phenotype to be queried. Pooled gRNA-infected cells are screened by microscopy and individual cells exhibiting the phenotype that was screened are identified, photoactivated, and isolated via flow cytometry. To identify phenotype to genotype connections, the gRNA lentiviral integration site is sequenced in single cells from the selected sorted cells. We explored the regulation of the transcription factor TFEB. In response to the cellular metabolic state, TFEB initiates lysosomal and autophagy transcriptional programs. Using AIMS, we screened for factors involved in TFEB translocation in or out of the nucleus. In addition to several known hits, novel TFEB regulators were also detected. These results demonstrate the feasibility of AIMS. Our approach is not only a novel implementation demonstrating how machine learning can be used to to explore cell biology, but, this new platform also enables phenotypic-based screening at the subcellular level, an approach which has been largely unavailable.

P50/B51

Designer Membraneless Organelles Equip Eukaryotic Cells with a Second Genetic Code to Enable Orthogonal Translation of Selected Messenger RNAs.

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Genetic code expansion (GCE) is a powerful tool to study and control protein function with single-residue precision in a variety of organisms ranging from *E. coli* to mice. It is widely used to perform labeling for super-resolution microscopy, photocontrol cellular function, or to introduce site-specific posttranslational modifications. This is achieved by introducing an orthogonal tRNA/synthetase suppressor pair into the living host, to recode a stop codon to incorporate a noncanonical amino acid (ncAA) into the nascent chain. This technique is codon-specific, but it cannot select specific mRNAs, so other naturally occurring stop codons could be suppressed leading to potential interference with housekeeping translation. Nature avoids unwanted cross-talk between cellular processes by confining specific functions into organelles. We aimed to design an organelle dedicated to protein engineering. Inspired by the concept of phase separation we hypothesized that such an organelle could be designed membraneless. Phase separation can generate high local concentrations of proteins and RNAs in cells and has recently gained attention owing to its role in the formation of specialized organelles such as nucleoli or stress granules that perform complex and specific functions inside the cell. We combined phase separating proteins (FUS/EWSR1 or SPD-5) with microtubule motor proteins (KIF13A or KIF16B) to generate orthogonally translating organelles in living cells that contain an RNA-targeting system, the stop codon suppression machinery and ribosomes. These large organelles enable site- and mRNA-specific ncAA incorporation, decoding one specific codon exclusively in the mRNA of choice. Our results demonstrate a simple yet effective approach to the generation of artificial organelles in eukaryotic cells. These semi-synthetic cells are capable of simultaneously executing two genetic codes, one canonical one for untargeted proteins in the cytoplasm and one expanded genetic code for chosen mRNAs inside of the synthetic organelles, thereby providing a route towards customized orthogonal translation and protein engineering.

P51/B52

A Novel Human Uterine Leiomyoma Model for Studying Mechanisms of Fibrosis Induced by Environmental Estrogens.

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Background: Uterine leiomyomas (fibroids) are highly prevalent benign tumors in women of reproductive age, which often become progressively fibrotic with excessive accumulation of extracellular matrix (ECM) components. Fibroids are estrogen-responsive and may be the target of environmental estrogens. Bisphenol a (BPA) and its analogues (BPS, BPAF) are ubiquitous environmental estrogens, which have been shown to promote fibrosis in rats and mice. The role of environmental estrogens in fibrotic changes in human uterine fibroids is unclear. A deeper understanding of the mechanisms of fibrosis in fibroids requires model systems that closely mimic the in *vivo* tumors. As a result, a 3D human fibroid culture system is being explored as a possibly more realistic system to study the effects of environmental estrogens on fibrosis development. **Objectives:** to develop a human uterine leiomyoma (ht-UtLM) 3D cell culture system to evaluate the role of environmental estrogens (BPA, BPS, and BPAF) on the induction and molecular mechanisms of fibrosis. **Methods:** Ht-UtLM 3D cell cultures

(spheroids) were developed and assessed for viability and microscopic characteristics. Cell proliferation in the 3D model was tested using cell counting kit-8 assays after treatment with BPA, BPS, or BPAF (10^{-6} - 200 μM) for 24, 48 and 72 hours. Differential gene expression was determined at 24 hours using RT² Profiler™ PCR arrays of ECM and fibrosis genes. Fibrosis was evaluated by a Masson's Trichrome stain and western blotting targeting ECM components at 7 days. Network and pathway analyses were done using Ingenuity Pathway an alysis (IPA), and activation of an identified pathway was confirmed by western blotting. **Results:** the 3D ht-UtLM spheroids had a cell viability percentage of 89.77%. BPA, BPS, and BPAF all increased cell proliferation and gene expression including subtypes of collagen genes (COL1A1, COL15A1, and COL16A1), and regulators of fibrosis (TGFB, TGFBR, and SMAD). Compared to controls, fibrosis was more evident in spheroids treated with BPA, BPS, and BPAF at 7 days. ECM components including collagen, fibronectin and versican were increased with all three treatments. BPA, BPS, and BPAF all upregulated expression of profibrotic genes, predicting the activation of the TGF-beta pathway in these profibrotic effects. Phosphorylation of TGFBR1 was increased significantly after treatment with BPA, BPS or BPAF for 3 hours, confirming the activation and involvement of the TGF-beta pathway. **Conclusions**: the 3D fibroid spheroid culture system is an effective and valuable model for studying fibrosis in human uterine fibroids. BPA and its analogues, BPS and BPAF, can promote fibrosis in uterine fibroids through TGF-beta signaling, and may pose as a health risk to women with uterine fibroids.

P52/B53

Building an Image an alysis Pipeline to Measure the Effect of TNF-α on HSV-1 Infected Cells.

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Computer vision is a set of tools for extracting quantitative information from images in a systematic, reproducible, and unbiased manner. In the context of biological imaging, a typical image analysis pipeline often consists of binary masking, single-cell segmentation, feature extraction, and tracking over time. Here, we construct such a pipeline in Python and apply it to a dataset of HSV-1 (Herpes Simplex Virus-1) infected NIH3T3 fibroblasts treated with TNF-α (Tumor Necrosis Factor). The experimental setup included wells with HSV-1 either present or absent and treated with different TNF-α concentrations. The time-lapse experiment was carried out to investigate the role of TNF-α in inducing apoptosis in HSV-1 infected cells. The pipeline was used to identify all the cells in the microscope images taken for a particular experimental condition. It was then used to track the quantitative properties of identified cells throughout the course of the time-lapse experiment. We find that the image analysis pipeline performs well with over 70% of cells in each frame being successfully tracked. The processed data shows that higher TNF-α concentrations dramatically increase the rate of death in HSV-1 infected cells. This data will then be used to fit a death-proliferation model that predicts if infected cells will undergo apoptosis based on TNF-α concentration and infection with HSV-1.

P53/B54

Pomegranate: a Volumetric Segmentation and Reconstruction Pipeline for Fission Yeast.

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Fission yeast (*Schizosaccharomyces pombe*) is an excellent model organism to study basic eukaryotic cell biology. A feature of fission yeast is its well understood rod-shaped geometry, where cells grow via tip

elongation as they progress through the cell cycle. This feature can be leveraged for the accurate detection and segmentation of cells, as well as for calculating accurate approximations for cell volume. Automated microscopy and image analysis tools have been developed over the past decade with a variety of approaches to cell detection, segmentation, and data collection for fluorescence microscopy images. We have developed a segmentation pipeline, Pomegranate, that uses a combination of bright-field images, fluorescent markers, and the known morphological characteristics of fission yeast to produce a volumetric fit of a cell's whole cell and nuclear geometry. A volumetric fit allows for the analysis of individual regions in a three-dimensional reconstruction of the sample, providing information on the concentration and localization of a protein of interest. In conclusion, Pomegranate proves to be a useful extension to existing image analysis tools for fission yeast, offering a semi-automatic single cell analysis workflow optimized for high density images.

P54/B55

Fast and Predictive 3d Neuron Reconstruction for Light Microscopy Images.

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The human brain is composed of approximately 100 billion neurons that collaborate to interpret our senses and control our thoughts and actions. Neuron type, localization and connectivity all contribute to the complex processes required for every organism with a nervous system to function. Thus, systematically reconstructing the 3D morphology of neurons is essential to further advance our understanding of how the brain functions. In the last five years, the BigNeuron project (1) has ran several comparative tests using 30 distinct algorithms for automated 3D neuron reconstruction. The tests were primarily ran using small data sets containing a single neuron or a few sparsely distributed neurons. While these tests were useful at showcasing the pros and cons of each algorithm, many real-world challenges remain when attempting to reconstruct neurons in large and densely populated data sets. We have developed an enhanced voxel scooping algorithm and deployed it in four distinct modes: 1) fully automatic with soma detection, 2) fully automatic without soma detection, 3) semi-automatic path prediction and 4) semi-automatic point-to-point. The last two modes can be used in parallel. To further facilitate the creation of neurons using the semi-automatic modes we have created a dynamic camera mode which follows new traces as they are validated, and we have added an auto-clipping mode which gives users the option to render the data in the region the user is working on. We validated our novel approach on a large data set (1200x1200x2613) depicting a mouse brain section which had been optically cleared using PEGASOS (2) and imaged with a Leica SP8 confocal microscope or the Cleared Tissue LightSheet (CTLS) system (3). Initial tests show that the fully automated version takes 48 mins to complete producing 395 neurons, 815 dendrites, and 325,541 microns in total path length. Thus, the automatic approach can trace approximately 400 mm per hour. The same data set would take an estimated 12 workdays (assuming each day a human expert would manually trace neurons for an average of 5 hours). The estimation is based on 5 hours of human manual tracing which resulted in 33 completed neurons (24,156 microns in total path length), representing a throughput of 4.8 mm per hour. In comparison, when using the semi-automated modes mentioned above, we could trace at a pace of 46 mm per hour. We demonstrated that the automated method was 8.7x faster than the semi-automated method. Moreover, the automated and semi-automated modes are 83x and 9.5x faster in comparison to manual tracing, respectively. We are now testing this approach on even bigger and denser optically cleared data sets imaged with the CTLS.

P55/B56

Quantitative Workflow for Segmentation and Tracking of Large Numbers of Human Induced Pluripotent Stem Cells from Transmitted Light Microscopy Images.

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Label-free single cell segmentation and tracking of human induced pluripotent stem cells (hiPSC) from widefield (2D) transmitted light microscopy images could allow for monitoring dynamic cellular processes such as the growth and division of single cells under minimal light exposure conditions. However, segmentation and tracking of cells in such images is challenging because iPSCs are densely packed and distinguishing cell edges in the resulting images is difficult. Classical segmentation techniques failed to meet the required single cell segmentation accuracy on phase contrast or brightfield image modalities. Instead, we used convolutional neural networks (CNNs) with the U-Net architecture to segment single cells within the colonies. Training sets for the U-Net model were generated using the nuclear envelope fluorescence of the LaminB-GFP iPSC line (LMNB1) from the Allen Cell Collection. Classical image analysis algorithms accurately segmented the high contrast, fluorescent images, allowing for abundant availability of training data. We compared the accuracy of two U-Net models: one trained on phase contrast images and the nuclear mask generated by FogBank segmentation and the other trained on brightfield images and the same nuclear mask. Segmentation was most accurate when the network was trained on the phase contrast image modality. The image analysis pipeline consists of segmentation followed by a tracking algorithm (e.g. NIST Lineage Mapper) that identifies mitosis and recognizes merger events when two nuclei cannot be segmented. Importantly, all the reference data for developing and testing the pipeline was acquired with automated equipment and scalable methods, thus facilitating the collection of CNN training data when new segmentation models are required. The application of the workflow for quantifying single cell gene expression dynamics in gene edited iPSCs will be described.

P56/B57

Deep Learning Minimizes the Impact of Fundamental Microscopy Limitations.

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Fluorescence microscopy has contributed to numerous major discoveries in life sciences. This is despite the limitations all imaging systems have. Typically, light microscopes excel at just one of the three core factors that modulate image quality and/or sample viability: spatial resolution, temporal resolution and light exposure. To minimize the impact of the mentioned handicaps, deep learning (DL) enabled microscopy image restoration is starting to be adopted (Content Aware Image Restoration (CARE)(1) and our own work (2)). Here we illustrate how a customized Residual Channel Attention Network (RCAN)(3) can be used for image restoration for data created on either an instant structured illumination microscopy (iSIM) or a point (resonant) scanning confocal microscopy. 3D live cell imaging of mitochondria and lysosomes using iSIM: training was done using 20 3D image pairs depicting cells with Tom20-labelled mitochondria and 20 3D image pairs with Lamp1-labeled lysosomes. All 3D images were 1.9K x 1.5K x 14 voxels. The raw input images (RII) were captured using 3.4 W/cm^2 laser and the ground

truth (GT) data was acquired using 0.37 kW/cm^2 laser. Routine live cell iSIM imaging is done at ~ 100 W/cm2 - which significantly limits the length of recordings. The trained model was applied to 3D+time (500 time points) acquired at low laser power (3.4 W/cm^2) that was not used for training. Applying the trained model to new RII significantly improved signal to noise ratio (SNR), spatial resolution and overall appearance versus the RII. Fast image acquisition of cleared 3D samples using a confocal system in resonant mode: Training images were created with a confocal microscope with a 40x 1.3 NA objective lens and resonant scanner in single-line mode (RII) or 64-line average mode (GT). Acquiring GT data took >3x longer than acquiring the equivalent raw images. The RII had low SNR and included major pixel shift artifacts, normal for resonant scanning. The GT had high SNR and no noticeable artifacts. Training was done using 8 3D image pairs depicting fluorescently labelled neurons. Applying the trained DL model to new RII resulted in high SNR and spatial resolution images. We demonstrate that DL can greatly diminish the amount of light the sample is exposed to, allowing for a dramatic extension in experiment duration while retaining the capability to perform super resolution imaging. We demonstrate this capability by studying the dynamic interaction of mitochondria and lysosomal vesicles using iSIM. In addition, we discuss how the same type of approach can be adopted to significantly reduce the time needed to image optically cleared brain samples when using a point scanning confocal system equipped with a resonant scanner, while maintaining the spatial resolution.

P57/B58

GPU-accelerated Machine Learning-powered 3d Image Segmentation at Scale.

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Despite the recent advances in bio-imaging, the extraction of meaningful insights from large multi-dimensional microscopy images continues to be a major bottleneck to the advancement of science. Non-machine learning tools use manually engineered algorithms to generate results. They are inflexible and require a user to master several user-facing parameters before one can efficiently use the tools. By contrast, machine learning (ML) based solutions, only require the user to draw a few regions representing the object types of interest, are starting to be adopted by researchers as they are much easier to master. However, ML based image processing approaches suffer from a different drawback, they are computationally expensive and thus benefit from processing optimizations so that they can be used at scale. Here we discuss enhancements made to our ML-based image segmentation solution, Aivia's Pixel Classifier. We have implemented a wide range of 3D filters optimized for 3D data. Moreover, we have implemented a new train and apply pipeline which uses GPU, instead of CPU processing. The new GPU-accelerated ML-based image segmentation approach was compared to our previous state of the art ML-based image segmentation framework presented at this conference in 2018[1]. We used a test image with the following (xyz) dimensions: 607x531x61, 32 bit. Computer environment used: Windows 10, 16 GB RAM, NVIDIA GeForce GTX 1060 GPU, i7-6700HQ CPU @ 2.6 GHz. In two class segmentation, the GPU accelerated solution achieves 13 times speed up (55 seconds vs. 4 seconds). Moreover, RAM usage was reduced by 35x (7 GB vs. 0.2 GB). Next, we will benchmark the new approach using multiple image sizes as well as >2 segmentation classes. In conclusion, we found that the new solution was ~13x faster while requiring 35x less memory (RAM). This will allow researchers to benefit from the ease of use of ML-based image segmentation approaches while being able to process large data sets and/or multiple data sets.

New Techniques in Genomics and Proteomics: CRISPR

P58/B59

Crispr-cas9 Engineered Isogenic Luciferase Expressing Cell Lines as in *Vitro* and in *Vivo* Models for Cancer Research.

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The CRISPR-Cas9 system provides a robust gene-editing tool for basic research in biology and for the development of diseases models for translational research. The objective of this study is to use advanced technologies including CRISPR-Cas9 and bioluminescence to generate novel human cell lines for use as both in *vitro* and in *vivo* models in cancer research. Approximately 50% of melanoma patients have the $BRAF^{V600E}$ mutation and often become resistant to current BRAF inhibitors after several months of treatment. $KRAS^{G13D}$ is an acquired mutation associated with resistance to these inhibitors. In this study, CRISPR-Cas9 was used to knock-in the $KRAS^{G13D}$ point mutation into the A375 malignant melanoma cell line, which also contains the targetable $BRAF^{V600E}$ mutation. The resulting $KRAS^{G13D}$ mutant isogenic line A375, which has been validated at the genomic, transcript, and protein bio-functional levels, exhibits significant resistance to the BRAF inhibitors Dabrafenib and Vemurafenib when studied both in traditional 2D and 3D cell culture. Based on the in *vitro* model described above, we developed additional models for use in live-animal bioluminescence imaging by introducing a stable luciferase reporter into the isogenic A375 and $KRAS^{G13D}$ A375 cell lines. Both the relative and absolute bioluminescence signals within the cells were quantified and found to emit $4.9x10^5$ photons/cell/sec and $3.5x10^5$ photons/cell/sec, respectively. A subcutaneous xenograft model was utilized in this study and the in *vivo* live bioluminescence signal was quantified using the Xenogen IVIS imaging system to correlate tumor growth with luciferase expression. Both A375-Luc2 and $KRAS^{G13D}$ A375-Luc2 grew as subcutaneous tumors with increasing levels of bioluminescence when injected into nude mice. In addition, a portfolio of 5 human isogenic luciferase reporter cell line pairs and 18 human and mouse luciferase reporter cell lines were developed for the study of various cancer types. In conclusion, the combination of two technologies CRISPR-Cas9 technology and stable luciferase expression allows for the generation of isogenic luciferase expressing cell lines, which are valuable tools for elucidating mechanisms involved in tumorigenesis and for studying drug responses in *vitro* and in *vivo*.

P59/B60

Tissue-Specific Knockout of Genes in Mice Using DECAI CRISPR.

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Although it is easy to produce mice with a point mutation using CRISPR, it is much more difficult to make a mouse with the two loxP sites required for tissue specific knock out. Several specific methods for generating floxed mice by CRISPR have been developed to improve the low efficiency of correct dual loxP insertion but they are either technically challenging (microinjection into two-cell-stage mouse embryos, sequential injection) or do not work much better (long ssDNA donor containing both loxP sites with two guide RNAs) than the standard CRISPR method. A different approach which they called DECAI (DEgradation based on Cre-regulated-Artificial Intron) was recently taken by Bürckstümmer's group. Using human stem cell lines, they inserted a small artificial intron via CRIPSR into an exon found in all

isoforms of the protein they were trying to knock out. This intron has loxP sites on each side of its branch point and without Cre, splices itself out of the mRNA, leading to normal protein production. The intron also has 3 STOP codons in different frames so when the branch point sequence is removed by Cre, one of the STOP codons is put in-frame, in turn leading to degradation of the mRNA and knocking out protein production. As we are interested in how the protein BHC80 encoded by the gene *Phf21a* is involved in the epigenetic control of spermatogenesis, we decided to try this method in mice to ultimately knock out *Phf21a* in the presence of Cre in Sertoli cells. Using a system we developed earlier that quickly and closely predicts the mutational outcome of CRISPR treatment in mice using blastocysts, we tested how efficiently the artificial DECAI intron would be inserted by CRISPR. In this procedure, DNA is PCRed from mouse blastocysts previously injected as zygotes with the same CRISPR components intended to produce the engineered mice, then Sanger sequenced. Initial results are very promising, with over 35% of the blastocysts showing evidence of perfect insertion of the artificial DECAI intron and therefore a floxed *Phf21a* gene.

P60/B61

Development of an Imaging Based Pooled CRISPR Screening Platform.

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Recent development of CRISPR based screening technology provides new ways to quickly examine gene behavior on a genome wide scale. However, such screens generally require the application of a selection pressure to the entire cell population to identify a specific phenotype, mostly an extreme life and death phenotype. These requirements prevent its application to lots of interesting scenarios especially when morphological feature changes which does not lead to immediate cell death. To overcome this disadvantage, we developed a screening platform which preserves the power inherited from pooled format CRISPR screen, but utilizing microscopy detection followed by FACS to isolate specific cell population. To prove its feasibility, we applied this method to screen for regulators of nuclear size and identified 17 potential hits. This approach combines the strength of both pooled format CRISPR technology and imaging technology for easy and accurate phenotype identification, which could be widely applied to different cell biology researches.

P61/B62

An Easy CRISPR Regulator and Editor System.

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CRISPR has been developed as an important tool for DNA and RNA editing. Our all-in-one CRISPR system integrates the gRNA and Cas9 into one vector in order to fix the transfection molar ratio as 1:1. Using this system, we have developed several genome-wide CRISPR kits, such as CRISPR KN (Knockout), CRISPRa (activation) and CRISPRi (inhibition), and also CRISPR v3.0 dimerization system. Our CRISPRa/i are based on dCas9 platform, which can specifically regulate target promoter activities. Individually, our CRISPRa system is a modified SAM system, which displays a highly-efficient stimulating activity, and CRISPRi, built on the backbone of dCas9-KRAB-MeCP2, is able to act as an ideal inhibitor to target genes. Furthermore, our CRISPR v3.0 dimerization system is designed to couple different effectors to dCas9 platform upon the treatment of drug APA. This system could provide inducible gene regulation with lower off-target effects and better background control. In conclusion, our all-in-one CRISPR platform is an effective tool for DNA and RNA editing application.

P62/B63

Multiplex Genome Editing of Human Natural Killer Cell via Optimized CRISPR-Cas9 RNP Transfection.

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Natural Killer (NK) cell is an effective arsenal in immune system against malignant cells, and a promising cell type for immunotherapy. Activation of NK cell is an intricate process that involves the interaction between a series of NK cell surface receptors and target cell ligands, providing positive and negative signaling to ultimately determine the activation status of NK cells. A robust and precise genetic tool will be useful to dissect this complex mechanism and to allow engineering of NK cells with enhanced cytotoxicity against malignant cells. Currently, the mainstream genetic engineering of NK cell still relies on viral transduction, which is inefficient due to NK viral resistance and imprecise because of random viral integration. By leveraging the power of nucleofection, we are able to efficiently deliver pre-assembled CRISPR-Cas9:sgRNA ribonucleoprotein complexes (Cas9 RNP) into primary NK cells for multiplex genome editing. Parameters of nucleofection were extensively optimized for maximal Cas9 RNP editing, including buffers, nucleofection programs and dosage of Cas9 RNP. The genetic knockout efficiency of human NK cell is up to 90% while maintaining the viability at 90%. Multi-gene knockout in one-shot nucleofection is also achieved. We further established an expansion procedure for cryopreserved human primary NK cells. The expanded primary NK cell possesses intact NK cell markers and can be engineered by our optimized Cas9 RNP nucleofection protocol. Our results demonstrate the experimental feasibility of human primary NK cell sourced from frozen stock, when PBMCs from fresh blood is not easily accessible. Collectively, our work facilitates biological research and therapeutic engineering of human NK cell for off-the-shelf immunotherapy.

P63/B64

Novel Reagent for Cas9 Ribonucleoprotein Delivery into Stem Cells.

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The CRISPR/Cas9 genome-editing platform is a versatile and powerful technology to efficiently create genetically engineered living cells and organisms. This system requires a complex of Cas9 endonuclease protein with a gene-targeting guide RNA (gRNA) to introduce double-strand DNA breaks (DSBs) at specific locations in the genome. DSBs are then repaired by either the error-prone Non-Homologous End Joining (NHEJ) or the more precise homology-directed repair (HDR). NHEJ leads to randomly insertions and/or deletions (indels) which disrupt the targeted locus whereas HDR relies on the presence of a donor template for the precise sequence modifications. The success of CRISPR genome editing experiments in stem cells is limited by the intracellular delivery and expression of Cas9 protein and gRNA. Many methods for achieving CRISPR mediated genome editing have been identified and the choice of DNA, RNA or ribonucleoprotein (RNP) format is dictated by experimental goal and cell type. Multiple studies have found that CRISPR/Cas9 components delivered using RNP are associated with lower off-target genome editing when compared to other methods. However, the lack of techniques that can efficiently deliver RNP with low cytotoxicity in stem cells limits the use of RNP for genome editing in these cell types. To overcome this limitation, we performed an RNP delivery optimization and screened a large candidate of cationic polymer and lipid combinations in two human induced pluripotent stem cells (iPSCs) lines. The novel formulations that we identified from the screen offer low toxicity and superior RNP delivery in iPSCs when compared to leading commercially available

transfection reagents. Moreover, this formulation can be used to deliver DNA oligo donors for HDR genome editing. The ability to safely and efficiently deliver RNP into stem cells will enable a wide range of scientific studies from gene expression modulations to drug discovery screening.

P64/B65
Genome Editing Platform Using Engineered Cas9, Which Has the Enhanced Pam Flexibility and Dna Specificity.
L. Min, 20850, M. Liu, Y. Shi, P. Wei, X. Liu, Y. Wang; OriGene Technoligies, Inc, Rockville, MD.

Genome Editing Platform using Engineered Cas9, which has the Enhanced PAM Flexibility and DNA Specificity Li Min, Mingjuan Liu, Yuhong Shi, Ping Wei, Xuan Liu, Yiran Wang CRISPR-Cas9 nucleases have already been widely used for genome editing. Cas9 and other enzymes need a protospacer adjacent motif (PAM) adjacent to the targeted site. The most commonly used SpCas9 needs NGG 3' end of the targeted sequence. NGG occurs frequently in GC-rich genomes, in every 42 bases of human genome. However, gene editing with SpCas9 will be limited for AT-rich regions. To solve this problem, SpCas9 mutants were explored for broader PAM recognition. The engineered Cas9 was reported to have broad PAM recognition and increased specificity. The engineered Cas9 was tested in our CRISPR mammalian genome editing system and demonstrated the Enhanced PAM Flexibility and DNA Specificity.

Actin and Actin-Associated Proteins 1

P65/B67
Nuclear Actin Polymerization Regulates Transcription through Facilitating Rna Pol Ii Phase Separation.
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Transcription controls essential cellular processes including selective genes expression and cell differentiation. A variety of proteins are involved in the regulation of RNA Pol II transcription. Nuclear actin, as one of these regulators, is critical for the transcription of RNA Pol II, but the molecular mechanisms remain unknown. The phase separation regulation mechanism during transcription has been uncovered in the last two years, revealing the important role of phase separation in maintaining chromatin structure and regulating transcription. In this work, we studied the regulation of nuclear actin on RNA Pol II from the perspective of phase separation. Through establishing a live cell imaging system to control the phase separation process of RNA Pol II in real time and a sensitive and interference-free nuclear actin labeling method, we found that nuclear actin is involved in the regulation of transcriptional processes by controlling RNA Pol II phase separation through interacting with multiple proteins, including PSF, Nono, N-WASP, and Arp2/3 complex. Importantly, we also demonstrated that signaling such as serum stimulation caused polymerization of nuclear actin, enhanced the interaction between unstructured regions of RNA Pol II, and promoted RNA Pol II phase separation. Using the new phase separation concept to explain the long-conflicted nuclear actin regulation of transcription has opened up a new perspective for the study of nuclear actin function, increase our understanding of transcriptional regulation, and guide us to understand more phenomena in the cell from the view of phase separation.

P66/B68

The Role of Src Kinase in Regulation of Dynamin-2 Activity at Podosome.

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Podosomes are Src kinase regulated and actin-rich structures critical for cell adhesion, migration and invasion. While the membrane remodeling GTPase dynamin-2 has been found to be enriched at podosomes to mediate adhesion molecule endocytosis as well as to regulate the bundling of actin and the stiffness of podosome, the mechanism orchestrating the function and recruitment of dynamin-2 to podosome is still unclear. Given that dynamin-2 is a substrate of Src kinase, we hypothesize that Src kinase phosphorylation on dynamin-2 may play an important role for its activity at podosome. We thus investigate the effect of dynamin-2 mutants on the Src phosphorylation sites with cell biology and biochemistry approaches. We found that both the phospho-mimetic and phospho-deficient mutants of dynamin-2 have significant impacts on dynamin 2-actin association both in C2C12 myoblast and in *vitro*. Furthermore, the Src-mediated phosphorylation also affects the GTP hydrolysis activity of dynamin-2. Together, our findings demonstrate that Src kinase phosphorylation on dynamin-2 regulates its function in podosome.

P67/B69

Deconstructing the Presynaptic Actin Cytoskeleton.

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Actin is the most abundant cytoskeletal protein at presynaptic terminals, where it is heavily implicated in many functions, ranging from vesicle mobilization and traffic to morphogenesis and stability of the synapse. However, little is known about how diverse types of actin assemblies are organized and coordinated at these sites. Here, we aim to characterize components and regulators of actin cytoskeleton (other than actin itself) in presynaptic terminals at the *Drosophila* larval neuromuscular junction (NMJ), a model synapse that offers unique access for studying synaptic biology in the animal. For this purpose, we down-regulated 23 actin-associated proteins (AAPs) in the fly nervous system and examined their effects on actin assemblies, using genetically encoded fluorescent actin and actin-labeling markers. Neuronal down-regulation of AAPs (including actin nucleators, capping proteins, stabilizers, network regulators, cross-linkers, actin-based myosin molecular motors) affected the levels as well as the distribution of F-actin at the synapse. In particular, a prominent synaptic subpopulation of actin patches exhibited complex changes in both number and size upon downregulation of conserved AAPs, molecules associated with the spectrin submembraneous cytoskeleton, as well as myosin motors. In parallel, we studied the effect of the down-regulation of the AAPs on synaptic morphology, and on the active zone and periactive zone at the NMJ. Overall, we identified unique and overlapping molecular players in the organization of different functional components of the synapse. In sum, our results present first in *vivo* screen of presynaptic actin regulators/components and point to potential regulators of different actin assemblies, affecting distinct synaptic structures/functions. For future studies, we aim to obtain more detailed picture on individual AAPs, and their contribution to the stability and function of presynaptic actin assemblies.

P68/B70

Myosin18aα Targets the Rac Gef β-pix to the Dendritic Spines of Cerebellar Purkinje Neurons to Promote Actin-dependent Spine Maturation.

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Dendritic spines are signaling microcompartments that serve as the primary site of synapse formation in neurons, and that house the machinery underlying memory formation. Actin assembly and myosin 2 contractility play critical roles in the maturation of spines from filopodial precursors. Myosin 18A is a myosin 2-like protein expressed from flies to man that lacks motor activity, is sub-stochiometric to myosin 2, and co-assembles with myosin 2 to make mixed filaments (Billington et al., 2015). Myosin 18A is alternatively spliced to create multiple isoforms that contain unique N- and C-terminal extensions harboring both recognizable and uncharacterized protein: protein interaction domains. These observations suggest that myosin 18A serves to recruit proteins to mixed filaments of myosin 2 and myosin 18A. One such protein is the Rac/Cdc42 guanine nucleotide exchange factor (GEF) β-PIX, which is known to promote spine maturation by activating the nucleation promoting factors WAVE and WASp, leading to Arp2/3-dependent branched actin filament assembly (Zhang et al., 2005; Saneyoshi et al., 2008; Yan et al., 2013). Here we show that myosin 18Aα is highly expressed in cerebellar Purkinje neurons and concentrates in spines along with myosin 2 and F-actin. Myosin 18Aα's spine targeting is driven by both co-assembly with myosin 2 and an actin binding site present in its N-terminal extension. miRNA-mediated knockdown of myosin 18Aα results in a significant defect in spine maturation that is manifested as increases in spine length and density, and that is rescued by an RNAi-immune version of myosin 18Aα. Importantly, β-PIX co-localizes with myosin 18Aα in spines (but not when its myosin 18Aα binding site is deleted), and its spine localization is lost upon myosin 18Aα knockdown. Moreover, myosin 18Aα knockdown results in a significant reduction in the amount of F-actin per spine. These and other data argue that mixed filaments of myosin 2 and myosin 18Aα present within Purkinje neuron spines form a complex with β-PIX that promotes the conversion of filopodia precursors into mature spines by activating Arp2/3-dependent branched actin filament assembly downstream of β-PIX's GEF activity.

P69/B71

Simulating the Actin Cytoskeleton during Bleb Nucleation.

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Blebs are round membrane protrusions that are involved in important cellular processes such as cell motility, cytokinesis, and apoptosis. Recent studies have focused on the importance of cells that use blebs for cell migration in 3D fibrous environments. Blebs are initiated either by a local delamination of the actin cortex from the membrane due to loss of membrane-cortex adhesion proteins or by a local disruption of the actin cortex. In either case, the cortex degrades in the region of bleb initiation and then reassembles in the bleb. Myosin contractility has been shown to be necessary for bleb formation. It has further been hypothesized that a localized increase in myosin contractility on the cortex results in a rupture of the actin network that can lead to bleb nucleation. A minimal model consisting of actin filaments, myosin molecular motors, and actin cross-linker proteins is used to quantify conditions where

holes, or defects, can develop in an initially isotropic cytoskeletal network. The model is simulated using the Cytosim computational platform. Results show that increased myosin fosters actin alignment, while increasing the concentration of actin cross-linkers decreases the pore size of the cytoskeletal network. Holes in the actin network develop at relatively high concentration of both cross-linkers and myosin. Simulation results suggest that a spatially localized defect, representing cortical actin network architecture during bleb nucleation, requires localized actin depolymerization rather than localized distributions of cross-linkers and myosin.

P70/B72

The Drosophila Melanogaster Rab Gap RN-tre Plays a Role in Regulating Non-muscle Myosin Ii Localization and Function.

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To identify novel regulators of non-muscle myosin II (NM II) we performed a targeted RNAi screen using a stable Drosophila melanogaster S2 cell line expressing EGFP-tagged regulatory light chain (RLC) of NM II and mCherry-Actin. This screen yielded a single hit, the Rab-specific GTPase-activating protein (GAP) RN-tre. Using total internal reflection fluorescence (TIRF) microscopy, we found that RNAi depletion of RN-tre led to a loss of NM II RLC localization in a manner that phenocopied depletion of proteins in the Rho pathway. This loss of localization corresponded to a loss of function as well—depletion of RN-tre led to an increase in actin retrograde flow rates and a decrease in S2R+ cells' ability to constrict in a cellular contractility assay. We next wanted to determine if RN-tre's regulation of NM II is dependent on its GAP activity. Interestingly, over-expression of constitutively active Rabs 5, 6, and 19, all of which are thought to be inactivated by RN-tre, failed to alter NM II RLC localization. Instead, we found that expression of constitutively active Rho, Rho-kinase (Rok), and the phosphomimetic NM II RLC could each rescue the loss of localization following RN-tre depletion. Furthermore, we assayed the cells for phosphorylated RLC by immunostaining and found a substantial decrease in phosphomyosin-positive cells following RN-tre depletion. In addition, depletion of RN-tre led to a decrease in the amount of active Rho as compared to controls. Collectively, our results suggest that RN-tre plays an important regulatory role in NM II RLC distribution, phosphorylation, and function, likely through conserved Rho signaling and is an example of cross-talk between the secretion machinery and actomyosin contractility.

P71/B73

Active Rho G-proteins Are Necessary for the Recovery of Damaged Hair Bundle Mechanoreceptors in *Nematostella Vectensis*.

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Hair bundle mechanoreceptors located on the tentacles of sea anemones become disorganized when the animals are immersed in calcium-free seawater. Such disorganization causes loss of function of the hair bundle mechanoreceptors. However, organization returns to the hair bundle mechanoreceptors once the animals are immersed in calcium-containing seawater. As part of the normal recovery of the morphology (and function) of the hair bundles, reorganization of the actin cytoskeleton occurs within the stereocilia of the hair bundles. In previous studies, Rho G-proteins have been localized to the stereocilia of hair bundles and found to be involved in actin polymerization. This study aims to

determine whether active Rho G-proteins are critical during recovery of hair bundles from damage caused by immersion in calcium-free seawater. This study includes the use of Rho inhibitors, phalloidin staining, and various types of microscopy (i.e., phase-contrast, oblique, and fluorescence). Following a 1-hour immersion in calcium-free seawater, there is a significant decrease in the abundance of hair bundles at the tips of tentacles. Over the course of a 6-hour recovery period in calcium-containing seawater, a partial recovery in abundance of hair bundles occurs within 3 hours, but a full recovery does not occur within 6 hours. During the recovery period, hair bundles significantly widen at the tips within 2 hours of recovery before returning to tip widths comparable to untreated controls within 6 hours. Additionally, stereocilia of hair bundles become significantly longer compared to untreated controls within 2 hours of recovery before returning to lengths comparable to untreated controls within 6 hours. Following the addition of Rho inhibitors to the calcium-containing seawater, progress in the recovery of abundance and morphology of hair bundles is hindered. Neither recovery of abundance of hair bundles nor elongation of stereocilia occurs within the 6-hour recovery period in the presence of Rho inhibitors. These data suggest that active Rho G-proteins are necessary for the normal recovery of abundance and morphology of hair bundle mechanoreceptors damaged by immersion in calcium-free seawater.

P72/B74

Cell Cycle and Cytoskeletal Dynamics during *C. Elegans* Muscle Progenitor Migration.

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Defects in cell cycle regulation or morphogenic movements are associated with many pathogenic processes and are regarded as hallmarks of cancer metastasis. Here we explore the migration and differentiation of mesodermal precursor cells in the nematode *C. elegans* as a model for cell cycle and cytoskeletal regulation of a morphogenic movement. The *C. elegans* sex myoblasts (SMs) are derived from progenitors that migrate during larval development and then proliferate and differentiate into the adult vulval muscles. The two SM founder cells travel anteriorly during the second larval stage until they flank the gonad. Upon arrival, the SM cells undergo three rounds of cell division before terminally differentiating into uterine and vulval muscle. Several signaling pathways and guidance cues important for SM specification, migration, and differentiation have been identified by classical methods, but the cell biological mechanisms underlying SM migration and pre-differentiation remain largely unknown. To explore cellular mechanisms, we used high-resolution, time-lapse microscopy to analyze the behaviors of SM cells in *vivo*. We show that these cells generate numerous actin rich filopodia and exhibit complex cytoskeletal dynamics throughout their lifetime. To further investigate the role of cytoskeletal dynamics during SM pre-differentiation we are performing an RNAi screen of candidate genes that we hypothesize may regulate SM migratory and protrusive behaviors. Furthermore, using a newly developed cell cycle state sensor we establish that SMs are in G0 quiescence when they migrate, and re-enter the cell cycle upon arrival. These findings lay the groundwork for establishing *C. elegans* SMs as a cell-biological model for cell migration and protrusion formation in *vivo* to study the relationship between migration, protrusion, cell-cycle state and cytoskeletal regulation.

P73/B75

NRIP Not Only Affects Myogenesis Differentiation but Also Acts as a Novel Actin Binding Protein for Myotube Fusion.

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NRIP is a Ca^{2+}-dependent calmodulin-binding protein and responsible for muscle contraction. To investigate NRIP role in muscle development, firstly, we found that NRIP could be expressed in various tissues such as muscles, heart, brain at embryonic stage; and NRIP expression profile in limb muscle tissues started at embryonic stage during muscle development, and peaked at postnatal P1 and P7, and mildly less expression at 6 weeks. To characterize NRIP role in myogenesis, we found that primary myoblasts from muscle specific NRIP knock out mice significantly exhibited the deficit differentiation and myotube fusion, the results also found in NRIP-null C2C12 cells. Fistly, to illustrate NRIP function in myogenesis differentiation, the muscle specific NRIP knock out mice revealed the delayed satellite cells activation/proliferation and differentiation; that further supports NRIP involved in myogenesis differentiation. Intriguingly, we found that NRIP is a novel actin binding protein and located at membrane. In our previous study, NRIP global knockout (gKO) mice show higher frequency of small myofiber size distribution than wild-type mice at day 21 post cardiotoxin-induced injury during muscle regeneration, indicating that NRIP may regulate myoblast fusion to form myofiber. Myoblast fusion has been reported to be strongly associated with actin cytoskeleton remodeled by actin-binding proteins. To examine NRIP role in myotube formation, NRIP was found to locate and accumulate at cell contact site of myoblast-myoblast and myoblast-myotube (containing two nuclei), and NRIP colocalized with actin to form podosome-like structure. By using time-lapse microscopy analysis revealed the dynamic changes of the actin cytoskeletal remodeling between NRIP and actin in C2C12 during myoblast fusion in which at initial (0-30 min) the actin and NRIP-enriched foci in cytoplasm of C2C12, and then (60-180 min) the foci protruded toward cell membrane to form a podosome-like protrusive structure which might participate in invasion during myoblast fusion. Moreover, NRIP was characterized for direct participation of fusion process using cell fusion assay (C2C12-KO19); indicating that NRIP could increase the proportion of fused myotube with more nuclei. Taken together, NRIP not only affects myogenesis differentiation but also acts as a novel actin binding protein for myotube fusion.

P74/B76

Cytoskeletal Tension Regulates Cell Growth and Proliferation.

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Cell mechanical behavior plays crucial roles in growth, proliferation and disease progression. In particular, cytoskeletal tension may modulate intracellular biochemical signaling pathways, and affects cell growth and proliferation through mechanotransduction mechanisms. Here, using Y-27632, a ROCK (Rho-associated protein kinase) pathway inhibitor, we uncover novel relationships between cytoskeletal tension, protein synthesis, cell cycle and cell volume. We show that Y-27632 significantly suppresses phosphorylation of myosin light chain and YAP nuclear translocation. We discover that cell shape changes dramatically and cell volume decreases gradually by 20% in 2 hours upon Y-27632 inhibition in both HT1080 and NuFF cells. Furthermore, we show that protein synthesis rate is down-regulated upon Y-27632 inhibition, with a response time longer than cell volume decrease. In addition, we find that inhibiting myosin assembly and cell tension slows down cell cycle progression from G1 to S. These

results suggest that cell cytoskeletal tension not only acts as a regulator of cell migration and differentiation, but also influences cell cycle, cell growth and proliferation.

P75/B77

Molecular Mechanism for Differential Force-regulated Actin Binding by Vinculin and α-catenin.

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Cells in the body must perceive and appropriately respond to mechanical stimuli in their local microenvironments during development and to maintain homeostasis. Dysfunction of mechanical signal transduction (”mechanotransduction”) pathways is correspondingly implicated in a wide variety of human diseases, including metastatic cancer, muscular dystrophy and cardiomyopathy, yet therapeutics targeting these pathways are largely absent due to the lack of knowledge of the underlying molecular mechanisms. The actin cytoskeleton plays a central role in mechanotransduction through the regulated interactions between actin filaments (F-actin) and more than 150 actin-binding proteins (ABPs). Research at the cellular and tissue levels have implicated mechanical regulation of actin-ABP interactions in mechanotransduction, but how force regulates critical actin-ABP interactions at the molecular level is largely unknown. Here we have employed biophysical and structural biology techniques to address this question. Using a novel myosin-motor based TIRF reconstitution assay, we find that a pair of homologous ABPs which are essential for cell-cell and cell-matrix adhesion, α-catenin and vinculin, have distinct mechanosensitive F-actin binding. The actin-binding domain (ABD) of α-catenin preferentially engages F-actin in the presence of mechanical load across actin filaments (“mechanoaccumulation”), while vinculin’s ABD does not. Simultaneous optical trapping and confocal microscopy experiments demonstrate that a load of ~1 pN across single filaments activates α-catenin ABD binding. Atomic resolution cryo-EM structures of the metavinculin ABD-F-actin (2.9 Å) and α-catenin ABD-F-actin (3.2 Å) complexes demonstrate both ABDs undergo major conformational changes upon actin engagement, prominently at their N- and C-termini, and their C-terminal regions differentially refold to bind distinct sites on the filament surface. A C-terminal truncation of α-catenin’s ABD constitutively binds F-actin regardless of force, and a chimeric protein of vinculin’s ABD featuring α-catenin’s flexible termini gains mechanoaccumulation activity, suggesting the α-catenin C-terminus-F-actin interaction is necessary and sufficient for mechanically regulated binding. This work, for the first time, establishes a force-regulated actin-binding mechanism in structural detail, and lays the groundwork for the rational design of therapeutics targeting cytoskeletal mechanotransduction pathways.

P76/B78

Modular Lim Domains Are Direct Sensors of Actin Strain to Mediate Mechanotransduction.

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The LIM domain is a protein-interaction module that is implicated in cellular mechanosensation through the actomyosin-adhesion system. However, the molecular mechanism by which LIM proteins transduce mechanical signals remains elusive. Motivated by the hypothesis that mechanical deformations of actin filaments could serve as an upstream signal in mechanotransduction, we performed an imaging-based

screen for LIM-domain proteins that accumulate on stress fibers in cells exposed to cyclic stretch ("mechanoaccumulate"). Three LIM-domain protein families, FHL, paxillin, and zyxin, emerged as mechanosensors. We identified a highly conserved phenylalanine present in mechanosensor LIM domains, and demonstrated that mutating this phenylalanine in each LIM domain abolishes the mechanoaccumulation of LIM proteins on stress fibers. We developed an in vitro actin force reconstitution assay wherein actin filaments are exposed to forces generated by surface-immobilized myosin motors, which reveals that strain in single actin filaments is necessary and sufficient for FHL, paxillin, and zyxin binding. FHL2 is a transcriptional coactivator which has been reported to translocate to the nucleus in soft environments to upregulate its transcriptional targets. We hypothesized that force-induced conformational changes in F-actin could generate binding sites for FHL2 in stiff microenvironments, serving as a platform for retaining FHL2 in the cytoplasm and preventing its nuclear shuttling. We found that our FHL2 mutant exhibits enhanced nuclear enrichment compared with wild-type in cells plated on stiff substrates. Furthermore, live cell imaging reveals nuclear translocation of wild-type FHL2 upon cytochalasin D treatment to disrupt F-actin and its return to the cytoplasm upon drug washout, consistent with our hypothesis. Our results demonstrate a novel mechanotransduction mechanism mediated by the direct binding of FHL proteins to strained actin.

P77/B79

GFP-based F-actin Orientation Probes for Fluorescence Polarization Microscopy and Speckle F-actin Orientation Imaging in Living Cells.

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Fluorescence polarization microscopy (FPM) is useful for detecting both positions and orientations of fluorescently labeled molecules and is thus expected to provide novel insights into the architectural dynamics of labeled cellular proteins. For example, when F-actin is appropriately labeled with fluorescence, FPM enables us to resolve the spatial organization of F-actin in its dense networks, and even the orientation of a single actin filament can also be determined. Although observation of actin with FPM has been commonly performed by fluorescent dye-conjugated phalloidin, there are several disadvantages for its use in live-cell FPM e.g. requirement of cumbersome microinjection and stabilization of actin filaments caused by phalloidin which may compromise the physiological actin dynamics. These disadvantages have greatly limited the application of FPM to live-cell imaging of actin, therefore novel actin probes for FPM are required. GFP-based actin probes, such as Lifeact-GFP, have been widely used, and some of them have been reported to have relatively small effects on the actin dynamics, but the fluorescence polarization of these probes does not reflect the orientation of actin since a flexible linker is usually used to connect the fluorescent protein to the actin-binding molecule. To develop GFP-based actin probes suitable for FPM, we rigidly connected circular permutated green fluorescent protein (cpGFP) to the N-terminal α-helix of actin-binding protein Lifeact or utrophin calponin homology domain (UtrCH), and normal mEGFP to the C-terminal α-helix of UtrCH. We expressed these constructs in cultured cells and evaluated their performance in FPM observation. Among them, some of the mEGFP-UtrCH constructs labeled actin filaments anisotropically, and showed low cytosolic background signal, enabling our successful speckle F-actin orientation imaging. We

conclude that these mEGFP-UtrCH constructs would be useful actin probes for F-actin orientation imaging with FPM, including speckle F-actin orientation imaging.

P78/B80

Structural Insights into Actin-binding Mechanisms Using Novel Fluorescence Technology for Spectrin Superfamily Members.

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We seek to understand the molecular mechanisms by which cytoskeletal proteins of the spectrin superfamily bind to actin, with the ultimate goal of therapeutic discovery for a wide range of cytoskeletal and neurological disorders. Recently we found that a disease-causing missense mutation, L253P, in the actin-binding domain (ABD) of β-III-spectrin, causes a 1000-fold increase in actin affinity. The high conservation of L253 in other spectrin family members suggests that this residue has a conserved role in regulating actin binding. The objective of this project was to test whether residues at the equivalent position of L253 regulate actin binding in other ABDs. To test this we developed a novel FRET biosensor that monitors the binding of spectrin family members to actin in live cells. Using this assay, we found that mutation of the equivalent residue (M240P) in α-actinin-4, a founding member of the spectrin superfamily, does not increase actin binding. Structural analyses suggest that β-III-spectrin L253 coordinates a lysine residue (K65), which forms a salt bridge between the two calponin homology (CH) subdomains comprising the ABD. In α-actinin-4, this salt bridge is absent, with alanine in place of lysine. We hypothesize that lysine 65 is an important structural residue in β-III-spectrin that negatively regulates actin binding, together with leucine 253. To test this, lysine 65 was mutated to alanine, and actin affinity of the mutant determined by FRET in live cells and in *vitro*. Co-sedimentation assays showed that the K65A mutant ABD has higher F-actin binding affinity (Kd = 4.2 μM) than the wild-type ABD (Kd = 75 μM). FRET assays confirmed increased actin binding in live cells. Further, circular dichroism spectroscopy showed that the K65A mutation destabilizes the ABD, similar to L253P. This suggests that increased actin binding caused by the K65A mutant reflects an opening of the CH1-CH2 interface. These data indicate that lysine 65, together with leucine 253, negatively regulates actin binding in β-III-spectrin. Moreover, this study demonstrates that lysine 65 represents a key structural difference between β-spectrin and α-actinin. This study paves the way for structure-based drug discovery for treatment of disorders involving actin-binding proteins in the spectrin superfamily, such as spinocerebellar ataxia type 5, cardiomyopathies, and focal and segmental glomerulosclerosis.

P79/B81

Single-molecule Characterization of F-actin Velocities in Living Cells.

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Active flows within the actin cytoskeleton generate mechanical forces that allow cells to migrate, change shape, and exert mechanical forces on their surroundings. In theory, the distribution of F-actin velocities should contain rich information about the structure and dynamics of the local actin cytoskeleton. However, most previous measurements report only average F-actin speeds. This limitation reflects in large part the low signal-to-noise ratio of typical F-actin tracking measurements, as localization errors on individual fluorescent fiducials are often comparable to the distances traveled between frames. Here, we describe a to our knowledge novel analysis that circumvents this issue by the deconvolution of

measurement noise from the measured velocity distributions of individual F-actin fiducials. This analysis yields F-actin velocity distributions that, unlike means, allow us to test physical models of force propagation through the actin cytoskeleton. We anticipate that this approach may be useful in the analysis of other single-molecule measurements that face limitations imposed by low signal-to-noise ratios.

P80/B82

Vrp1/WIP Activates Wsp1/WASp Nucleation Promoting Factor Activity at Sites of Endocytosis in Fission Yeast.

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Arp2/3 complex-mediated branched-actin nucleation is important across species for cell motility and endocytosis. Branched actin assembly is stimulated by Nucleation Promoting Factors (NPFs), such as Wiskott-Aldrich Syndrome protein (WASp). WASp works in complex with WASp Interacting Protein (WIP), which protects WASp from degradation in mammals. Human patients with mutations in either WASp or WIP develop Wiskott-Aldrich Syndrome, a serious immune disorder. An important unresolved question is how branched actin assembly machinery is regulated and positioned to efficiently drive membrane deformation. We investigate mechanisms of branched actin assembly in a favorable model system, endocytic actin patches in fission yeast *S. pombe*. Here WASp homologue Wsp1 remains stable in the absence of WIP homologue verprolin Vrp1, providing an ideal environment to study WIP/Vrp1 function. Wsp1 and Vrp1 exist in a transient complex with myosin-1 Myo1 where Wsp1 and Myo1 activate the Arp2/3 complex to form the branched actin network necessary to internalize endocytic structures. *S. pombe* Vrp1 enhances Myo1 NPF activity in *vitro*, but its physiological role remains unknown. To uncover the role of Vrp1 on actin patch assembly in *vivo*, we generated internal domain deletions in the *vrp1*$^{+}$ gene, expressed these mutations under the endogenous promoter in cells expressing endocytic proteins tagged with fluorescent mGFP, and examined the effects of these mutations on endocytic patch dynamics using quantitative spinning disk confocal microscopy. We found Vrp1 localization to sites of endocytosis is entirely dependent on its C-terminal WASp Binding Domain (WBD). Additionally, using an in *vivo* protein binding assay, we discovered the Vrp1 Proline Rich Domain (PRD) directly binds the Myo1 tail, independent of Wsp1. When this Wsp1-Vrp1 interaction is disrupted by removing the Vrp1 WBD, the number of actin molecules localized to sites of endocytosis is significantly reduced. Surprisingly, removal of the Myo1-interacting PRD or the actin binding WH2 domains did not impact actin patch accumulation, suggesting Vrp1 activates Wsp1 NPF activity independent of the PRD and WH2 domains. This reduced accumulation of actin correlates with a reduction in the distance endocytic vesicles internalize, suggesting Vrp1 activation of Wsp1 provides force needed for membrane deformation. Further, using a novel in *vivo* actin polymerization assay, we observed the Vrp1 WBD, but not the PRD or WH2 domains, was necessary to enhance Wsp1 mediated actin assembly. This data provides novel evidence that Vrp1 regulates endocytosis by activating Wsp1 NPF activity.

P81/B83

CD2AP Links Actin to PI3 Kinase Activity to Extend Epithelial Cell Height and Constrain Cell Area.

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<META NAME="author" CONTENT="华硕"> Epithelial cells generate three different membrane domains (apical, basal, and lateral) that perform distinct functions. The ratios of apical and basal membrane to lateral membrane varies between cell types depending on physiological function, but little is known as to what controls the height of the lateral membrane relative to the area of the apical and basal domains. Here, we show that the actin-binding scaffold protein, CD2AP, is a critical determinant of epithelial proportions. Depletion of CD2AP or PI3-kinase inhibition results in loss of F-actin and expansion of apical-basal domains which comes at the expense of lateral membrane height in MDCK cells. Immunofluorescence staining showed that CD2AP co-localized with PI3K p85α. CD2AP is required for PI3K p85α and p110γ recruitment and PtdIns(3,4,5)P_3 synthesis along lateral membranes. Thermophoresis results showed that SH3 domains (1-329 a.a.) of CD2AP binds to the N-terminus of PI3K p85α (1-315 a.a.) which contains two proline-rich motifs. Targeting SH3 domains (1-329 a.a.) of CD2AP to lateral membrane was sufficient to restore p85α and p110γ membrane levels. CD2AP 1-329 a.a. or PI3K p110 γ membrane tethering both recovered F-actin accumulation and apical expansion in CD2AP-knockdown MDCK cells. SH3 domains of CD2AP contributes to both PH-GFP fluorescence signals generation along cell borders and constraining cell spreading area. Thus, CD2AP recruits PI3K to regulate cell height. We also showed that CD2AP is not actin monomer sequester, but directly associates with F-actin and regulates actin dynamics in *vitro*. Single filament elongation experiments showed that both NT- and CT- of CD2AP decreased actin polymerization rates. In *vivo*, Latruculin B treatment attenuated both CD2AP and PI3K levels, i.e. CD2AP and PI3K are both upstream and downstream of actin polymerization. Thus we hypothesized that CD2AP bridges actin assembly to PI3K activation to form a positive feedback loop to support lateral membrane extension. Our results may provide insights into squamous to cuboidal to columnar epithelial transitions seen in complex epithelial tissues in *vivo*.

P82/B84

The Chytrid Fungus *Batrachochytirum Dendrobatidis* Has Both an imal-like and Yeast-like Actin Networks.

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The frog parasite Batrachochytirum dendrobatidis (Bd) is a chytrid fungus with two main life stages, a motile zoospore stage lacking a cell wall, and an immotile zoosporangia stage with a chitin cell wall. We have previously shown that Bd zoospores move on surfaces using actin and Arp2/3 dependent cell protrusions called pseudopods. Because this motility is likely involved in pathogenesis, it is important to understand how pseudopods and other actin structures are regulated in Bd. To understand the possible mechanisms Bd may be using to control its actin networks, we used BLAST searches to determine the conserved set of actin regulatory proteins present in Bd and other chytrid fungi. We found that chytrids have an actin regulatory protein repertoire intermediate to that of animals and that of other fungi. For instance, chytrids encode the canonical actin nucleators Arp2/3 complex and formins, and, unlike other fungi, also encode the Arp2/3 regulator, SCAR/WAVE complex. Given chytrids' divergence before the diversification of fungi, this finding gives fundamental insight into the evolution of actin regulatory networks. Through a combination of phalloidin staining and small molecule inhibitors, we found that Bd

has temporally and phenotypically distinct actin structures which resemble either those of animals or of yeast. The finding of animal-like and yeast-like actin structures present in one organism proves useful in uniting the two main model systems of actin study. Furthermore, expanding the knowledge of actin-based motility in Bd brings us closer to finding a way to combat the devastating effects this pathogen has on amphibian populations worldwide.

P83/B85

To Elucidate Hyaluronan Mediated Motility Receptor Mediated Daughter Cell-size Control Pathways during Mitosis.

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Background: Cell division must evenly distribute genetic information between two daughter cells with equivalent size and cytoplasmic factors. These processes rely upon the position of the spindle during anaphase. Spindle position is regulated by dynein-dependent cortical pulling forces and, when the anaphase spindle is off-centered, asymmetric membrane elongation (AME). During AME, Ran-GTP at chromosomes is brought proximal to the cortex and induces rapid membrane blebs due to actomyosin-based contractile forces on one side of the plasma membrane, altering cellular boundary to correct daughter cell size. HMMR is known to regulate the establishment of proper spindle position. HMMR locates Ran-GTP to the centrosome as part of a polo-like kinase1 (PLK1)-dependent spindle positioning pathway; therefore, we investigated whether HMMR also regulates AME during cell division and affects daughter cell-size control. **Methods:** Overexpression and knockdown of HMMR in HeLa cells were achieved by tetracycline inducible system (tet-GFP-HMMR-HeLa) and siRNA, respectively. Live cell imaging was used to track membrane blebbing and daughter cell-size. IP-MS was used to identify potential actin-related binding partner of HMMR during mitosis. The localization and abundance of actin-binding proteins during mitosis were determined using super-resolution fluorescence microscopy. Candidate kinases known to regulate daughter cell-size control were modulated with sublethal (0.01 X IC50 dose) doses of small molecule inhibitors. **Results:** tet-GFP-HMMR-HeLa cells showed more frequent mispositioned anaphase spindles, a higher occurrence of AME during anaphase and unequal daughter cell ratios. IP-MS of HMMR during mitosis identified a set of actin-binding proteins, Arp3, NMIIb and MYO18A. Arp3 co-localized with HMMR at the spindle pole during anaphase and the cortical localization of Arp3 was affected in tet-GFP-HMMR-HeLa cells. Mild inhibition of Aurora a (but not PLK1, Aurora B, or CK1) was sufficient to dampen kinase activity at spindles poles, as measured by immunofluorescence, and also partially rescued Arp3 cortical recruitment, AME and daughter cell-size control in tet-GFP-HMMR-HeLa cells. **Conclusion:** GFP-HMMR overexpression during mitosis induces AME and deregulates daughter cell-size control. Mechanistically, the inhibition of Aurora a kinase partially rescued these phenotypes and Arp3 cortical localization implicating augmented kinase activity as a contributing factor downstream GFP-HMMR overexpression that deregulates AME and daughter cell size control. Direct interaction between HMMR and Arp3 is an additional putative factor that is currently under investigation.

Regulation of Actin Dynamics 1

P84/B86

How Do Cells Move in Our Body? Study of the Cellular Mechanisms Allowing Immune Cell Migration in Restricted Spaces.

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Upon infection, mature dendritic cells (mDCs) migrate from peripheral tissue to lymph nodes to activate T lymphocytes and initiate the adaptive immune response. This fast and tightly regulated process imposes a series of physical constraints and is tuned by different microenvironmental factors, such as the physical properties of the tissue[1]. Mechanistically, mDCs migration relies on acto-myosin flow and contractility, which are dependent on non-muscular Myosin IIA (MyoII) activity. However, the specific mechanoresponse that allows mDCs to adapt their migration machinery to irregular 3D landscapes has not been fully characterized. In this work, we combined a series of approaches, from micro-fabricated devices to ex vivo skin models, to dissect the cytoskeleton rearrangements used by mDCs to overcome the physical barriers imposed by the tissue microenvironment[2]. By using microchannels of different sizes, we have shown that mDCs are able to maintain a constant speed while migrating at different levels of confinement. This reveals the extreme capacity of mDCs to adapt their migration machinery in response to changes in the geometry of their microenvironment[3]. At the cellular level, confinement in microchannels induces a fast and specific acto-myosin remodelling in mDCs. Upon cell compression, polymerized cortical actin concentrate in two areas of the cell: 1) at the extreme back and 2) at the cell centre, where the microtubule organizing centre and the lysosomes localized. The structure at the back is associated with MyoII and is required to maintain mDCs speed in narrow and irregular landscapes. Interestingly, preliminary data shows that the polymerized actin structure at the cell centre is important to maintain cellular integrity while mDCs move in irregular 3D microenvironment. Altogether, our results reveal a complete acto-myosin rearrangement triggered by confinement, which is essential for mDCs migratory plasticity that allows these cells to move in intricate 3D geometries. The full understanding of how mDCs and other leukocytes adapt their motility to specific tissue structures will provide better knowledge on how cell migration is controlled in confined spaces and new insight to finely tune their migration to promote or prevent immune responses. 1. Vargas P., Barbier L., Sáez P. J. & Piel M. Mechanisms for fast cell migration in complex environments. Cell Dyn. 48, 72-78 (2017).2. Sáez P. J., Barbier L. et al. Leukocyte Migration and Deformation in Collagen Gels and Microfabricated Constrictions. In Cell Migration: Methods and Protocols (ed. Gautreau, A.) 361-373 (Springer New York, 2018). 3. Barbier L. et al. Myosin II Activity Is Selectively Needed for Migration in Highly Confined Microenvironments in Mature Dendritic Cells. Front. Immunol. 10, 747 (2019).

P85/B87

Actin Dynamics for Efficient Phagocytosis: Mechanosensitivity and New Regulators.

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Phagocytosis is a mechanism of internalization and degradation of micro-organisms or cellular debris. Phagocytosis is important for remodelling of tissues, disposal of dead cells and bacterial clearance. Actin polymerisation provides the force that drives the membrane deformation required to engulf particulate matter during phagocytosis. We identified new actin regulating proteins that are recruited at sites of membrane extensions and phagosome closure. We showed that they are crucial for efficient uptake of various particles and bacteria. Key to dissecting the mechanism by which this occurs, is understanding how the complex mechanosensitive machinery of actin binding proteins sense force and stabilize actin anchoring during phagocytosis, from initial receptor binding, through to phagosome formation and closure. A novel experimental approach utilizing traction force microscopy is being used to observe phagocytosing macrophages on substrates of biologically relevant stiffness. We show that the capacity of macrophages to perform efficient phagocytosis varies with the substrate stiffness. In addition, phagocytosing macrophages display different properties such as contractile energy and polarization degree as compared with non-phagocytosing cells. The mechanotransduction pathways implicated will be discussed.

P86/B88

Optical Flow Techniques Reveal Differences in Actin Wave Guidance Across Scales.

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Understanding the rearrangements of the cytoskeleton is essential to developing a complete picture of dynamic cellular processes, such as migration, division, and differentiation. Here we focus on the role of the extracellular environment as an important modulator of actin dynamics. In addition to often-studied biochemical cues, the extracellular environment also provides physical cues such as the topography of the extracellular matrix (ECM). We show that topographical cues are capable of guiding both cell migration and actin dynamics in multiple cell types. Periodic surface topographies with feature sizes comparable to those of in *vivo* collagen fiber networks guide actin dynamics bidirectionally. We show that such subcellular-scale guidance can be exploited to create unidirectional guidance of both actin and cell migration through periodically repeating local asymmetries. We find that for a given asymmetry, the preferred direction of both actin waves and motion is dependent on cell type. To quantitatively distinguish actin dynamics phenotypes in response to surface topography, we have adapted a computer vision algorithm, optical flow, which allows us to determine the direction of actin waves at the microscale (individual pixels). This quantitation allows us to compare two cell types with markedly different migratory modes and physiological purposes, slowly migrating epithelial MCF10A cells and fast migrating neutrophil-like HL60 cells. Though they have distinct morphodynamics, optical flow measurements indicate that both cell types exhibit guided one-dimensional actin waves (esotaxis) on periodic surface topographies. However, clustering the optical flow vectors into regions with similar flow directions shows that the mesoscale features of actin dynamics are cell type dependent. These results suggest that esotaxis is a common cellular process, yet the details of the esotactic response exhibit quantitative differences among cell types that can be exploited to achieve goals such as cell type dependent unidirectional guidance.

P87/B89

Mechanically Induced Activation of Actin Elongation Factor Dia1 at Focal Adhesions Protects the Cytoskeleton from Damage and Facilitates Stress Fiber Repair.

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Cells are truly the ultimate "smart material" regulating their mechanical properties dynamically and locally to match mechanical demands of their microenvironments. Such plasticity of cell mechanics relies on the regulation of the structure and dynamics of cell cytoskeleton. Contractile forces exerted by myosin II on actin filaments are known to regulate cytoskeleton dynamics, but the mechanism remains poorly understood. Here we show that myosin contractility regulates actin cytoskeleton dynamics by enhancing the activity of actin elongation factor Dia1 at focal adhesions, an integrin-based protein assembly that link cell cytoskeleton to the extracellular matrix. We show that this mechanism works as a 'safety valve' restricting mechanical tension on the stress fibers and protecting actin cytoskeleton from mechanical damage. By combining live-cell imaging and pharmacological manipulations, we assessed how suppression of major actin elongation factors affects the rate of actin polymerization at focal adhesions. We found that formin inhibition decreased polymerization by ~ 60%. By depleting individual formins, we identified Dia1 as the major actin elongation factor at focal adhesions. To understand the role of mechanical forces applied to formins we used pharmacological perturbation of myosin II to attenuate contractility within the cell. We found a dose-dependent decrease in actin polymerization at focal adhesions as myosin contractility was suppressed by ≈90%. The regulation of actin polymerization by myosin contractility was abolished in cells depleted of Dia1, indicating that contractility modulates Dia1 activity. By using a novel super-localization technique, we visualized instantaneous rate of actin polymerization at focal adhesions and showed that Dia1 activity is not constant but undergoes myosin-dependent cyclical events. To investigate the biological role of myosin dependent Dia1 activation, we assess how depletion of Dia1 affects the integrity and repair of actin cytoskeleton. We found that suppression of actin polymerization at focal adhesions increases the magnitude of spontaneous stress fiber damage and decreases the efficiency of zyxin-mediated stress fiber repair. As actin filaments are subject to complex mechanical constraints in living cells, these results provide important insights into how formins sense these mechanical constraints and regulates actin organization to prevent damage.

P88/B90

Spine Segmentation Is Regulated by Mechanical Coupling of the Notochord Sheath and Myosepta.

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In zebrafish, segmentation of the notochord epithelial sheath provides a template for osteoblast recruitment and vertebral bone formation in the developing spine. While notochord segmentation seems to follow a tissue-autonomous program, the precise positioning and size of its segments is influenced by interactions with the adjacent, tendon-like myosepta, which arise from the somite boundaries. However, how the interaction between tissues modulates notochord segmentation is unclear. To investigate how myosepta regulate notochord segmentation, we generated novel transgenic tools to monitor localization of mechanosensing proteins and contractile machinery in the notochord sheath. Using live-imaging approaches, we observed that initiation of notochord segments occurred next to myosepta and was accompanied by the nucleation of stable, tension-sensitive protein clusters. In contrast, fish with disrupted myosepta lacked segmented localization of these clusters and failed to

precisely space notochord segments. Furthermore, enrichment of mechanosensing complexes suggested that transduction of mechanical cues from the myosepta promoted assembly of contractile machinery in the notochord sheath. Live-imaging of newly developed reporters for non-muscle myosin and F-actin revealed the formation of cortical actomyosin networks in new and presumptive segments. When recruitment of contractile machinery was prevented in the notochord sheath, segment size and morphology was affected, suggesting that cortical forces within notochord sheath cells regulate tissue patterning. Together, these findings reveal that mechanical coupling of the notochord and the myosepta promote precise notochord segmentation through localized activation of intracellular cortical contractile networks.

P89/B91

A New Biomechanical Mathematical Model of Force Effects on Actin Dynamics during Clathrin-mediated Endocytosis.

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Background: During clathrin-mediated endocytosis, actin filaments assemble into a network that experiences and generates force. However, the interactions between the mechanical and chemical aspects of endocytic proteins remain unclear. This study proposes a new biomechanical approach to model how the actin filament network composition responds to different load forces in yeast. **Methods:** the biochemical component of the model was a system of ordinary differential equations representing key reactions involved in actin assembly/disassembly previously validated with in *vivo* yeast data. We added mechanical relationships to the reactions using alternative hypotheses of load force changes over time. The Arrhenius/Bell equation was used to specify an inverse relationship between load force and the actin polymerization rate constant. We assessed two different hypotheses. In the “constant load model”, the load force remains constant throughout endocytosis. In the “variable load model”, the polymerizing actin filaments increase the load force, creating a negative feedback loop: as more filaments polymerize, the load force increases, inhibiting polymerization. We simulated the time evolution of the components of the actin filament network including F-actin and capped filament ends (capping protein). We assessed model performance using two criteria: the number of peaks and the peak value of the time evolution of the copy number of each protein. **Results:** at load forces < 0.1pN, the F-actin time evolution had one peak under both models at forces > 0.1pN, the F-actin time evolution had two peaks under the “variable load model” - a pattern which is not seen in wild type but may be seen in mutant cells. Under the “constant load model”, the F-actin time evolution had one peak for a wide range of forces except for high forces when polymerization was completely inhibited. At forces < 0.02pN, both models had similar peak values for each protein and the F-actin and capped filaments/capping protein peak values were close to experimentally measured values. Under both models, the direction of change in peak value was the same and the peak number of polymerizing filaments, normalized to the peak number of branches monotonically decreased with increasing load force, explaining the monotonic decrease in F-actin. **Conclusion:** This new biomechanical modeling approach suggests that force feedback could possibly regulate actin assembly/disassembly and may vary with different load forces. In *vivo* experiments with varying load forces may allow us to confirm the force dynamics during the biochemistry of actin assembly/disassembly predicted by this model.

P91/B93

Arp2/3 Complex and Myosin2 Motor Activity Drive F-actin Mediated Microvilli Motility on Insulin Secreting INS-1 Cells as Revealed by TIRF-SIM Super Resolution Live Cell Imaging.

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Microvilli are cell surface protrusions consisting of a core bundle of actin filaments that are linked to the cortical actin network and plasma membrane via actin binding proteins. Epithelial cells generate arrays of microvilli known as brush border that function to increase solute uptake. Pancreatic beta cells also elaborate microvilli that serve as the primary site of the GLUT2 glucose transporter for glucose uptake. However, the dynamic changes that microvilli undergo during insulin secretion in pancreatic beta cells is not understood. We used super resolution TIRF-SIM microscopy to study the dynamics of microvilli during exocytosis in living pancreatic beta cells (INS-1 832/13) transiently transfected with mEmerald-LifeAct. Time-lapse sequences generated with TIRF-SIM microscopy showed that microvilli are highly motile structures and translocate at a mean velocity of 67 nm/s across the cell surface of INS-1 cells. To analyze the motile behavior of microvilli under low glucose conditions, individual microvilli were tracked and resulting trajectories were subjected to rose plot and mean square displacement (MSD) analysis. Rose plots showed that microvilli exhibit long-range displacements and MSD analysis generated a parabolic curve suggesting directed movement. Bayesian Hidden Markov Modeling (HMM) validated that microvilli undergo directed movement with both diffusive and transport states. Co-expression of mEmerald-LifeAct and mCherry-ARP2 in INS-1 cells showed that Arp2 was highly enriched in motile microvilli. To determine whether movement of microvilli was due to an Arp2/3-mediated assembly mechanism, Arp2/3 activity was inhibited with CK666. Trajectory analysis showed that inhibition of the Arp2/3 complex changed the motile behavior and that microvilli exhibited short-range rather than long-range displacements at a reduced mean velocity of 23 nm/s. Similarly, inhibition of myosin2 with blebbistatin also caused slow and short-range displacements of microvilli. MSD and HMM Bayes analysis for CK666- and blebbistatin-treated cells indicated an increase in the directed diffusive motion state for motile microvilli. Stimulation of INS-1 cells with 20 mM glucose to secrete insulin also reduced the average velocities and path lengths of moving microvilli compared to low glucose conditions. MSD and HMM Bayes analysis showed that stimulation with 20 mM glucose resulted in an increase in directed diffusive motion state compared to low glucose conditions. We, therefore, conclude that Arp2/3-mediated actin nucleation and myosin2 motor activity drive the movement of microvilli and we hypothesize that the movement serves to enhance their sensory function in response to changes of glucose levels in pancreatic beta cells.

P92/B94

Actomyosin Dynamics Tune Excitable Rhoa Activity to Produce Distinct Spatiotemporal Modes of Pulsed Contractility in the *C. Elegans* Zygote.

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Pulsed contractility is a widespread form of actomyosin contractility in which actin filaments and myosin II undergo local cycles of assembly, contraction and disassembly, driven by locally pulsed activation of RhoA. Spatiotemporal patterns of pulsed contractility vary widely across different organisms and developmental contexts, but the origins of this variation are poorly understood. In early *C. elegans*

embryos, pulses are driven by locally excitable RhoA dynamics involving fast positive feedback and delayed negative feedback mediated by F-actin dependent recruitment of the RHO GAPs RGA-3/4. Dynamic coupling of F-actin to RhoA inhibition suggests that modulating actin network assembly, contractility and disassembly could provide a way to access different spatiotemporal modes of pulsed contractility. To explore this, we quantified variations in spatiotemporal pulse dynamics produced by tuning levels of maternal factors that regulate actin network assembly, contraction, cross-linking and disassembly. Tuning actin network dynamics has minimal effects on the local rise and fall of RhoA activity during individual pulses, but can induce large variations in the pattern and local timing of pulse initiation, and in the spread of RhoA activity that determines pulse size and shape. Across many different perturbations, the initiation and spread of RhoA activity correlates with patterns of F-actin accumulation. An alysis of these correlations suggests that much of the variation in spatiotemporal RhoA dynamics can be explained by two effects: (a) that local F-actin depletion favors local pulse initiation and (b) that local regions of high F-actin density limit its subsequent spread. For example, perturbations which favor local actin depletion at the end of pulses, either through local disassembly or contractile instability, promote locally oscillatory pulse dynamics, with a dominant period set by the local persistence of F-actin accumulation. Decreasing overall F-actin levels and/or creating larger zones of F-actin depletion favors more rapid spread of RhoA excitation and larger pulses. Strikingly, when myosin activity and F-actin density are both reduced, RhoA activity spreads as traveling waves, as observed in other contexts. These observations identify local inhibition by F-actin as a major factor in shaping spatiotemporal pulse dynamics.

P93/B95

The Cell Cycle Regulates Excitability of the Actin Cytoskeleton.

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The actin cytoskeleton mediates a diverse array of cell shape changes, including cytokinesis. In *Xenopus laevis* (frog) and *Patiria miniata* (sea star) embryos, the actin cytoskeleton forms self-propagating waves of assembly and disassembly. Previous experimental results have shown that actin waves are preceded by waves of active Rho. Computational modeling suggests that these waves represent excitable dynamics of a reaction-diffusion system, with Rho behaving as the activator and F-actin behaving as the inhibitor. Strikingly, both wave sets are amplified by increased concentrations of the Rho GEF Ect2, a normal component of the centralspindlin complex and an essential cytokinetic regulator, suggesting a link between cortical waves and signaling from the spindle. We show that both Rho and F-actin waves are modulated by Cdk1 activity and are most apparent during cytokinesis phase of the cell cycle. Wave amplitude appears to be gated partially by the phosphorylation state of Ect2, and partially by cortical actin dynamics. We provide evidence that both of these factors feed into the mitotic regulation of cortical waves.

P94/B96

Actively-tuned Phase Transition in the Actomyosin Cortex.

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Phase transitions in biological contexts is thought to enable local enrichment reaction components, and has been suggested to contribute to construction of essential cellular architecture. F-actin filaments

make up the key architecture that is the cell's cortex. During the activation of the actomyosin cortex of the *C. elegans* embryo. Dynamic cortical F-actin puncta form and disassemble, with steady-state size distribution. The condensation of F-actin is preceded by nucleators WASP and Arp2/3.To study the dynamic clusters, the relative concentrations of WASP and F-actin over the lifetime of the puncta was tracked. The intensity evolution over the whole concentration landscape reveals that the growth rate of the cluster in WASP and F-actin is predicted by stoichiometry of the nucleators. The critical size of WASP condensates varies with F-actin levels. Condensation of WASP coupled to F-actin polymerization corresponds to cluster disassembly. Furthermore, the critical size of WASP condensates is tuned by F-actin condensation. Based on this relationship, we developed a model from which the direct rates can be measured from data. The model recapitulates the key characteristics of concentration evolution. This study reveals mechanisms that underly self-organization of protein condensates in the context of actin cortex remodelling.

P95/B97

The Huntingtin-interacting Protein Setd2/hypb Is an Actin Lysine Methyltransferase.

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SET-domain-containing-2 (**SETD2**) was originally discovered as Huntingtin Yeast Partner B (**HYPB**) due to its interaction in a yeast two-hybrid screen with the Huntingtin (**HTT**) protein. Later, SETD2 was identified as the methyltransferase responsible for trimethylation of histone 3 lysine 36 (**H3K36me3**), a mark of the histone code associated with active gene transcription. Most recently, SETD2 has been shown to bind microtubules and methylate α-tubulin at lysine 40 (**α-TubK40me3**) during mitosis and cytokinesis, with loss of SETD2 and a subsequent decrease in cytoskeleton methylation resulting in spindle defects and genomic instability. We have now discovered SETD2 associates with actin, another key component of the cytoskeleton, as part of an HTT complex containing the interacting partner **HIP1R**. HTT and its interacting partners regulate many actin-dependent processes, and we show here that as part of this complex, SETD2 functions as an actin methyltransferase. We found using in *vitro* methylation assays, the catalytic SET domain of SETD2 has intrinsic methylation capacity for actin, and antibodies directed against the trimethyl lysine epitope generated by SETD2 detected this mark on actin following in *vitro* methylation. To assess the relationship between SETD2 and actin methylation in cells, we used these SETD2 trimethyl epitope-specific antibodies to show that this epitope was present on actin from *SETD2*-proficient but not *SETD2*-deficient cells. The dependence on the SETD2-HTT-HIP1R complex for actin methylation was also demonstrated by knockdown of either *HTT* or *HIP1R*, which was sufficient to inhibit SETD2 binding and methylation of actin. SETD2 methylation was localized to areas of active cytoskeletal remodeling in cells, including the lamella/leading edge of migrating cells. Consistent with methylation at the leading edge, we identified a requirement for SETD2 in cell migration, and found biochemical and cellular evidence for an underlying F-actin polymerization defect following SETD2 loss. After its initial discovery as a Huntingtin-interacting protein, SETD2 has been the focus of studies related to its function on the epigenome. This work reveals for the first time an extended non-chromatin role for SETD2, and provides the first demonstration lysine methylation regulates the actin cytoskeleton. Localization of the SETD2 methyl epitope at the leading edge of cells, and disruption of actin polymerization and cell migration with loss of SETD2 points to a new role for SETD2 in regulation of the

actin cytoskeleton, expanding what is known about the key roles played by this methyltransferase in methylation of histones during transcription and spindle microtubules during cell division.

P96/B98

β-actin and γ-actin Nucleotide Coding Sequences Regulate Cell Migration Speed.

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β- and γ-cytoplasmic actins are ubiquitously expressed in every cell type and are nearly identical at the amino acid level, but play vastly different roles in *vivo*. Their essential role in embryogenesis is defined largely by the nucleotide sequence of their genes, rather than their amino acid sequence, however it is unclear which gene elements underlie this effect. Here we address the specific role of the coding sequence in β- and γ-cytoplasmic actins' intracellular function, using stable cell lines with exogenously expressed actin isoforms and their "codon-switched" variants. When targeted to the cell periphery using the β-actin 3'UTR, β-actin and γ-actin have differential effects on cell migration. These effects are directly coding sequence-dependent. Our results link these effects to the rates of actin isoforms' intracellular accumulation that likely affects the morphology and turnover of focal adhesions, and suggest that coding sequence-mediated actin translation plays a key role in cell migration.

P97/B99

Two Distinct Populations of Actin Filaments Have Differential Effects on Mitochondria in Response to Differing Stimuli.

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Over the years, mitochondria have graduated from being merely bioenergetic supply stations to discrete signaling organelles. Increasing evidence suggests that actin polymerization participates in mitochondrial communication and dynamics. In this study, we attempt to differentiate specific mitochondrially-associated actin filaments, their mode of organization and the effects they have on the mitochondria. We find at least two distinct modes by which actin and mitochondria interact. In one mode, elevation of cytosolic calcium activates endoplasmic reticulum (ER)-bound formin INF2, and the resulting ER-based actin polymerization stimulates two distinct events for subsequent mitochondrial division. First, an increase in transfer of calcium from ER to mitochondria results in inner mitochondrial membrane (IMM) constriction. Second, actin assembly promotes Drp1 recruitment to the outer mitochondrial membrane (OMM), which results in OMM constriction. In the other mode, dissipation of the mitochondrial proton gradient ('depolarization') causes an extensive 'cloud' of actin filaments around the depolarized mitochondria within 10 min. This actin assembly is dependent on the Arp2/3 complex but not on INF2. On a similar time scale after depolarization, mitochondria undergo extensive shape changes. Two aspects of these shape changes are surprising: 1) they are not primarily due to mitochondrial fission, and 2) they are not dependent on actin cloud assembly. The major component of the shape change is rearrangement of the IMM, resulting in a circularization of the IMM while maintaining the integrity of the OMM. Correspondingly, the shape changes depend upon the IMM protease Oma1, but not on the Drp1 GTPase. Actin clouds actually inhibit mitochondrial shape changes, suggesting that these clouds play a protective role for the depolarized mitochondria or for the rest of the cell. Actin clouds also suppress events downstream of depolarization, such as PARKIN accumulation. In summary, we show

that there are two distinct types of actin filaments assembly and they differ in their morphology, assembly mechanisms and effects on mitochondria.

P98/B100

The Formin Fmnl3 Participates in Depolarized Induced Actin Assembly Around Mitchondria.

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A growing number of studies suggests that actin polymerization participates in mitochondrial communication and dynamics in mammalian cells. We find that there are at least two distinct modes by which actin and mitochondria interact. In one mode (Chakrabarti et al (2019) *J. Cell Biol.* 217(1): 251-268), elevation of cytosolic calcium activates the endoplasmic reticulum (ER)-bound formin INF2, and the resulting actin polymerization stimulates two distinct events that promote mitochondrial fission: increased transfer of calcium from ER to mitochondria, (resulting in IMM constriction); and Drp1 recruitment to the outer mitochondrial membrane (OMM), resulting in OMM constriction. In the other mode (Fung et al (2019) *Biorxiv* 701771), dissipation of the mitochondrial proton gradient ('depolarization') causes rapid accumulation of an extensive 'cloud' of actin filaments around the depolarized mitochondria, dependent on Arp2/3 activity. Importantly, while this second mode is INF2-independent, multiple studies suggest that one of the remaining 14 formins is involved. By siRNA screen, we find that inhibition of FMNL3 in U2OS cells significantly reduces depolarization-induced actin assembly. In contrast, depolarization-induced actin polymerization is maintained upon knock-down of FMNL1, FMNL2, mDia1, mDia2, and INF2. The effect of FMNL3 KD is somewhat different from Arp2/3 complex inhibition, in that weak actin filament accumulation persists, but is greatly reduced and often does not encompass majority of mitochondrial network, a feature of depolarization-induced actin clouds. While FMNL3 participates in depolarized-induced actin assembly, it did not have any role in calcium-induced, INF2-dependent actin assembly. Mitochondrial depolarization also results in extensive mitochondrial shape changes that are due to reorganization of the inner mitochondrial membrane. These mitochondrial shape changes are independent of actin polymerization or Arp2/3 complex. In fact, pharmacological inhibition of Arp2/3 complex or knock-down of FMNL3 causes an increase in depolarization-induced mitochondrial shape changes. We propose that rapid actin polymerization around depolarized mitochondria acts as a protective mechanism, allowing for mitochondrial recovery.

P99/B101

Dynamic Organization of the Cortical Actin Network and Plasma Membrane Inner-leaflet through Action of Formins and Class I Myosin Motors.

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The outer membrane of the living cell is the interface that demarcates the cell and its environment. The chemistry of the plasma membrane is largely build up from lipids and proteins and local organization of this chemistry primes the cell to adapt and react to the external milieu. Experimental data [1] and a theoretical framework [2] have largely argued to view the plasma membrane and the closely juxtaposed dynamic actin-based cortical layer as an active composite. Cells consume energy to mechanically restructure this active composite by the action of actin filament nucleators and motors, which often bind to negatively charged phospholipids in the inner-leaflet to mediate remodelling of the cytoskeleton close to the plasma membrane. Here we focus on the role of class I non-muscle myosin molecular motors and formin actin nucleators in organizing the active composite at the plasma membrane. Both

formin and myosin I bind to negatively charged phospholipids at the plasma membrane and have the capacity to actively remodel actin filaments [3,4]. Using structured illumination microscopy to follow actin dynamics we find that formins and to a lesser extent myosin class I motors decrease the remodelling rates and dynamics of the cortical actin meshwork. Phosphatidylserine at the inner leaflet of the plasma membrane has been shown to be important for the trans-bilayer interaction of lipid-species at the outer leaflet, and its link to the dynamic cortical actin present at the inner leaflet. By using fluorescence correlation spectroscopy we find that levels of phosphatidylserine, at the inner leaflet, also regulates the organization and function of both formins and myosin I motor proteins, in turn helping to couple actomyosin activity and lipid organization at the plasma membrane. Together, our results provide evidence for a key role for both formins and non-muscle class I myosin motors in driving and maintaining the active composite that is constituted by the actin cortex interacting with the plasma membrane. References:[1] Goswami et al Cell 135 (2008), p10385-1097.[2] Gowrishankar et al Cell 149 (2012), p1353-1367.[3] Breitsprecher and Goode J Cell Sci 126 (2013) p1-7.[4] McConnell and Tyska Trends Cell Biol 20 (2010) p418-426.

P100/B102

Synergistic 330-foldPointed-end Actin Depolymerization by Cyclase-associated Protein and Cofilin.

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Living cells can rapidly disassemble and remodel actin networks in a few seconds, yet in *vitro* actin filaments take several minutes to depolymerize. Cellular mechanisms that can depolymerize actin this fast have thus far remained obscure. Using microfluidics-assisted TIRF and single molecule imaging, we have discovered a multicomponent system in which cyclase-associated protein (CAP) and Cofilin synergize to enhance actin depolymerization by 330-fold. Hexameric CAP molecules transiently interact with pointed ends of cofilin-decorated filaments and remove approximately 100 actin subunits per binding event. These are the fastest rates of actin depolymerization ever reported in *vitro*. We further show that CAP-cofilin synergy has been conserved across a billion years of evolution, from *S. cerevisiae* to mammals, and that it applies to both of the major isoforms of mammalian cofilin (Cofilin-1 and ADF). These findings establish a new paradigm, in which a filament end-binding protein and a filament side-binding protein work in concert to control actin dynamics, and explain how rapid actin depolymerization might be achieved in vivo.

P90/B103

Actin-membrane Detachment Is the Initiator of Polymerization-driven Protrusion.

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Actin polymerization generates cell protrusions by exerting force on the inner leaflet of the cell membrane. This occurs without the aid of molecular motors and is thought to involve “ratcheting” of the cell membrane via insertion of actin monomers between the f-actin network and the membrane. This process is aided by the arp2/3 complex, which nucleates filament branches from existing filaments, but requires membrane tethering of arp2/3 through the WASP/WAVE family of proteins. Thus arp2/3 functions both as an actin nucleating factor and as an actin-membrane linker that opposes monomer addition. The tethered Brownian ratchet model explains how a balance between tethered and free actin

monomers generates force during steady state protrusion, but cannot explain protrusion onset. Through a combination of experiments, computational analysis, and theoretical modeling, we show that local depletion of membrane cortex links that are distinct from those of WASP/WAVE initiates protrusion. Using live cell microscopy and time series analysis to measure thousands of protrusion events, we found that the membrane-cortex linker ezrin is depleted prior to protrusion onset and before enrichment of arp2/3. We also found that arp2/3 operates independently from ezrin depletion to drive protrusion, confirming that parallel, but independent mechanisms drive protrusion. Incorporating this information into a theoretical model for how actin polymerization generates protrusion, we found that small fluctuations in ezrin localization, but not actin, were sufficient to initiate protrusion. Model simulations also predicted that increases in ezrin affinity reduce protrusion frequency, duration, and velocity. Our experiments with ezrin mutants with such elevated affinity for actin confirmed these predictions. Altogether, our results demonstrate the requirements for different types of membrane cortex detachment during protrusion, enabling us to synthesize a more comprehensive model for how actin-driven protrusion generates force. Given that actin-membrane detachment is a critical regulatory factor in protrusions that do not require actin-generated force, our findings raise the intriguing possibility that morphologically different protrusions may be initiated by a common set of processes.

Myosins

P101/B104

Non-muscle Myosin IIc Tunes Actin Bundle Length in Microvilli.

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Transporting epithelial cells are covered in apical microvilli, which help to form a barrier between luminal contents and the cell. Microvilli are critical for maintaining cellular homeostasis, yet little is known about the mechanisms that control the morphology of these protrusions. The intestinal enterocyte is covered in densely packed microvilli, known as the brush border. Each protrusion contains a bundle of 20-30 actin filaments, with the barbed ends located at the distal tips, and the pointed ends anchored in a region of the cell known as the terminal web. Little is known about how the terminal web contributes to microvillar organization, morphology and maintenance. Within the terminal web, there are a variety of proteins, including non-muscle myosin II (NMII), a conventional myosin motor expressed in all eukaryotic cells. Within the human intestine, three different heavy chains of NMII are expressed; A, B and C, with NMII-A and NMII-C dominating expression in enterocytes. Here, we provide evidence that NMII-C forms a novel network across the enterocyte apical domain at the level of the terminal web. Line scans along the microvillar axis show that NMII-C is enriched near the pointed-ends of microvillar actin bundles. Our experiments reveal that altering the force production of NMII-C via genetic or pharmacological perturbations alters microvillar morphology. These findings suggest that NMII-C helps to regulate microvillar dimensions via the pointed end.

P102/B105

Non-muscle Myosin II Is Essential for the Negative Regulation of B Cell Receptor Signaling and B Cell Activation.

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B cells mediate humoral immunity and generate antibody responses against antigenic challenge. While positive signaling mediated through B cell receptors (BCR) is required for the initiation of B cell activation, prolonged or uncontrolled BCR signaling is associated with the rise of self-reactive B cells and the subsequent development of autoimmune diseases. We previously demonstrated a role for the actin cytoskeleton in the negative regulation of B cell signaling by driving B cell contraction after spreading on antigen-presenting surfaces, leading to the formation of BCR central clusters. Non-muscle myosin II (NMII) is an actin motor-protein that is expressed in B cells and known to be involved in B-cell migration, cytokinesis, and extraction of antigen (Ag) from antigen-presenting cells. This study reveals that NMII-mediated actin contraction negatively regulates BCR signaling using both NMII inhibitors and conditional knockout mice (NMIIA KO). Upon BCR binding of antigen, NMIIA is recruited to the spreading surface of B cells in contact with antigen-presenting surfaces and forms a ring-like structure upon B cell contraction. B-cell contraction and formation of BCR central clusters are delayed in both NMIIA KO B cells and blebbistatin-treated B cells. NMIIA KO and blebbistatin-treated B cells show up-regulation of BCR signaling, including tyrosine, CD79a, BLNK, and Erk phosphorylation when compared to control B cells. In contrast, the level of phosphorylated SH2-containing inositol 5-phosphatase (SHIP-1), an inhibitory signaling molecule, is reduced. Associated with this enhanced signaling, NMIIA KO results in increased numbers of spontaneously activated germinal center B cells when compared to control mice. Serum levels of Ag-specific IgG in response to immunization with a T-dependent antigen and binding affinity of IgG to the immunization antigen are reduced, while total serum levels of IgG are elevated in NMIIA KO mice compared to control mice. Furthermore, NMIIA KO mice displayed a reduced number of Ag-specific memory B cells compared to control mice. Together, our results reveal NMIIA exerts a B-cell-intrinsic inhibition on B-cell activation by promoting B cell membrane contraction, and coalescence of BCR microclusters into central clusters, revealing a novel mechanistic link between actin remodeling and negative regulation of signaling.

P103/B106

Nonmuscle Myosin 2 Regulates Cortical Stability of Vascular Sprouts during Blood Vessel Formation.

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Nonmuscle myosin 2 (NM2) plays important roles in regulating various basic cellular functions such as cell migration, cell adhesion and cell division. Three paralogs of NM2, namely NM2A, 2B and 2C, are expressed in mammals including mice and humans. Both NM 2A and 2B, but not 2C are essential for mice to thrive from embryonic development. To study the role of NM2 in blood vessel formation we ablated NM2 specifically in endothelial cells in mice. We find that NM2A plays a major role in regulating blood vessel formation. Ablation of NM2A results in a defect in back skin blood vessel formation with reduced blood vessel coverage and increased branching morphogenesis, while ablation of NM2B shows no obvious abnormalities. However mice with NM2B ablated together with one copy of NM2A show defects in blood vessel formation and mice with NM2A ablated together with one copy of 2B show more severe defects in blood vessel formation. Moreover homozygous mice expressing a motor-impaired

R709C-NM2B also show defects in blood vessel formation. Since NM2C is not detected in endothelial cells, these results suggest that both NM2A and 2B are involved in regulating blood vessel formation and that the mutant NM2B can interfere with normal NM2A function during blood vessel formation. In vitro vascular sprouting assays from the embryonic body (EB) shows that ablation of NM2A but not 2B results in excessive vascular sprouting. Live cell imaging of EB sprouting further reveals that the endothelial sprouts of the wild-type EB migrate persistently with numerous dynamic membrane blebs. Ablation of NM2A results in constant turning and branching of the sprouts. Moreover NM2A ablated sprouts show no obvious blebbing activity, but form large membrane protrusions indicating a loss of membrane stability. Live imaging of EGFP-NM2A EB sprouting further shows that NM2A is enriched during retraction of blebbing. We thus propose that NM2 regulates blood vessel formation by stabilizing the cortical membrane of vascular sprouts. We further show that ROCK is the major kinase which activates NM2 in vascular sprouting, since the sprouting defect occurs with inhibition of NM2 activity by blebbistatin or the ROCK inhibitor (Y27632), but not with the MLCK inhibitor (ML-7).

P104/B107

NM2A Deletion Promotes Cancer through Chromosomal Instability.

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Chromosomal instability (CIN) underpins tumor cell initiation and propagation. In human cancers, CIN correlates positively with poor patient prognosis. Recent studies implicate non-muscle myosin 2A (NM2A) for its role in cancer. Previously, our lab reported development of squamous cell carcinoma in the tongue epithelia of a murine model upon loss of NM2A. Here, we propose that NM2A functions as a tumor suppressor and loss of NM2A promotes genomic instability, aberrant growth, and dysregulated migration, leading to tumorigenesis. To investigate our hypothesis, the mitosis of human squamous cell carcinoma cells (UMSCC110) was characterized. UMSCC110 cells (a cell line developed at the Univ. Of Michigan) contain a preexisting mutation in both alleles of *Myh9* causing early termination of mRNA. The mutant NM2A null cells were transfected with a GFP-NM2A construct (lentiviral) to generate NM2A rescued cells. In these cells, histone H2B was labeled using red fluorescent protein (H2B-RFP-NM2A null and H2B-RFP-NM2A rescued cells) for visualization of mitotic events. In *vitro* analyses of cell models reveal differential rates of migration and growth regulated by NM2A. Live cell fluorescence imaging of cells undergoing mitosis were deconvoluted using Huygens Essential to visualize mitotic abnormalities such as anaphase bridging and lagging chromosomes. Time courses of mitosis between NM2A null (n=18) and NM2A rescued (n=17) cells were highly statistically different after two-way ANOVA analysis ($p < 0.0001$). On average, rescued cells completed mitosis more readily, with half lives of 102.6 ± 44 seconds compared to NM2A null cells with 82.20 ± 28.53 seconds. Similar levels of bridging chromosomes were detected in the NM2A null (8/18) compared to the NM2A rescued (7/17), however lagging chromosomes were observed only in NM2A null (7/18) but not in NM2A rescued (0/17) cells. An aphase lagging chromosomes are a primary chromosome segregation defect in tumors and their presence is indicative of underlying defects which cause chromosomal instability. Taken together, our data suggests NM2A functions as a tumor suppressor and loss of NM2A enables hallmarks of cancer.

P105/B108

Disrupted Mechanobiology Links the Molecular and Cellular Phenotypes in Familial Dilated Cardiomyopathy.

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Familial dilated cardiomyopathy (DCM) is a leading cause of sudden cardiac death and a major indicator for heart transplant. Familial DCM is frequently caused by mutations of cardiac sarcomeric proteins; however, connecting mutation-induced changes in sarcomeric protein function with the phenotype seen in cardiomyocytes is still a challenge for the field. Many of the changes in contractility at the molecular scale caused by sarcomeric DCM mutations are subtle, begging the question of what other factors could link molecular-scale changes with the cellular phenotype. We hypothesized that DCM-causing mutations of sarcomeric proteins affect not only sarcomeric contraction, but also how cardiomyocytes sense and respond to mechanobiological changes associated with aging and disease. To test this hypothesis, we studied the molecular and cellular consequences of a DCM mutation in troponin-T, ΔK210. Using biophysical and biochemical tools, we determined the molecular mechanism of ΔK210, and then we used computational modeling to predict that the mutation should reduce the force per sarcomere in cardiomyocytes. We used CRISPR/Cas9 to generate mutant human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs). We found that ΔK210 not only reduces the force per sarcomere in hiPSC-CMs, but it also causes cellular hypertrophy and impairs the ability of hiPSC-CMs to adapt to changes in substrate stiffness (e.g., myocardial fibrosis that occurs with aging and disease). These results link the molecular and cellular phenotypes and they implicate impaired mechanosensing by cardiomyocytes as an under-appreciated mechanism in the pathogenesis and progression of DCM caused by sarcomeric proteins. These results also have important implications for our understanding of cardiac disease and the design of precision therapeutics.

P106/B109

Altering the Backbone: a Mechanism for How Flightin-myosin Interaction Impacts Structure and Mechanics of Muscle Thick Filaments.

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Myosin II light meromyosin (LMM)-binding proteins potentially modulate thick filament structural and mechanical properties and influence muscle function. In *Drosophila* indirect flight muscles (IFM), flightin augments flexural rigidity of thick filaments, is necessary for the establishment of their in *vivo* length and for maintaining their structural integrity in active muscle. Flightin is defined by WYR, a ~52 amino acid domain conserved in hexapods and crustaceans. Loss of muscle function observed in *Drosophila* mutants lacking flightin is partially restored by truncated flightin missing either the N- or C-terminal domains flanking WYR and implicate WYR as essential for flightin function. In this study, we use circular dichroism to characterize the secondary structure of WYR and to uncover structural changes upon its complexing with LMM. Resultant ellipticity at 260-190nm reveals a structural profile for WYR and supports an interaction with the LMM that coordinates a conformational shift in both binding partners. The secondary structure of WYR is concentration and ionic strength dependent and displays predominant negative ellipticity at ~192nm with a 222/208 ratio of ~0.38, a trademark of a 3_{10} helix. WYR is found to be 40-50% unstructured with predominant beta character. WYR in the context of the LMM shows substantial conformation shifts including an increase in the 222/208 ratio. While the LMM

alone exhibits a 222/208 ratio of ~1.1, the LMM in the context of WYR exhibits a greater 222/208 ratio and is sedimented out of solution, maximally at 5:1. The increased 222/208 ratio is due to decreased intensity of the 208 minima, indicating increased coiled-coil content. Our results support the hypothesis that WYR binds the LMM and that this interaction brings about structural changes in the coiled-coil. LMM sedimentation by WYR suggests a possible mechanism by which flightin influences thick filament assembly in *vivo*. Furthermore, the distinct shifts in LMM secondary structure observed in the presence of WYR provide molecular insight into the role of flightin in defining the viscoelastic properties of the thick filament that influence muscle fiber mechanical output. Our results are further substantiated by high resolution cryo EM studies of IFM thick filaments that show the association of a non-myosin density with unwound portions of the myosin dimers in the filament backbone.

P107/B110

The Replacement of Myosin Molecules in Thick Filaments Is Different between Slow- and Fast-twitch Myofibers.

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Myosin, one of the myofiber-type markers, is a major myofibrillar component in skeletal muscles. Approximately 300 myosin molecules were assembled into a bipolar thick filament in the sarcomere. Our previous cell culture studies demonstrated that myosin molecules are vigorously exchangeable in thick filaments of myotubes, and that myosin replacement rate is modulated by myosin content in cytosol as well as heat shock protein (Hsp) 90 activity (Ojima K. et al., 2015, 2017, 2018). However, the effects of myofiber-types on myosin replacement remain unclear. In this study, we investigated the impact of myofiber-types on myosin replacement rate in isolated single myofibers. To examine this, we generated two types of fluorescence protein knock-in mice. One was GFP-Myh7 knock-in mouse expressing GFP-fused slow Myh7 (GFP-Myh7) instead of wild type Myh7. The other was KusabiraOrange-Myh1 knock-in mouse expressing KusabiraOrange-fused fast Myh1 (KusabiraOrange-Myh1) instead of wild type Myh1. In these mice, the expressed GFP-Myh7 and KusabiraOrange-Myh1 were precisely incorporated into the A-band of the sarcomeres. To measure the replacement rate of GFP-Myh7 in slow-twitch myofibers or KusabiraOrange-Myh1 in fast-twitch myofibers, fluorescence recovery after photobleaching (FRAP) technique was used. The fluorescence recovery degree in slow-twitch myofibers was higher than that in fast-twitch myofibers. The replacement rate of myosin molecules in fast-twitch myofibers was fast compared to that in slow-twitch myofibers. Next, treatment with inhibitors of protein synthesis or Hsp90 activity significantly reduced the fluorescence recovery degrees of myosin in both slow- and fast-twitch myofibers as compared to the control group. The inhibition ratios of fluorescence recovery were higher in slow-twitch myofibers than that in fast-twitch myofibers. Interestingly, the fluorescence recovery degree of slow-twitch myofibers treated with MG132, a proteasome inhibitor, was significantly lower than the control group, although that of fast-twitch myofibers was not affected. Taken together, our results suggest that the replacement of myosin molecules in thick filaments is different between slow- and fast-twitch myofibers due to the difference of myosin synthesis, Hsp90 and proteasome activity between them.

P108/B111

A Novel Isoform of Myosin 18A (Myo18Aγ) Is a Critical Component of Cardiac Sarcomeres.

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Two isoforms of myosin 18A (Myo18A), Myo18Aα and Myo18Aβ, are well known. Myo18Aα is widely expressed, whereas Myo18Aβ, which lacks the unique N-terminal extension of Myo18Aα, is predominantly expressed in hematopoietic cells. To elucidate the physiological functions of Myo18A we studied various *Myo18a* knockout mouse models. Homozygous deletion (homozygous tm1a, tm1b or tm1d alleles) in mouse was embryonic lethal at around embryonic day 11.5 (E11.5). Surprisingly, *Myo18a* was predominantly expressed in the embryo heart, and conditional deletion of *Myo18a* in cardiac myocytes was embryonic lethal. Neither Myo18Aα nor Myo18Aβ protein could be detected by Western blot in mouse heart using an antibody directed against the extreme C-terminus, whereas a larger than expected protein could be detected using alternative anti-Myo18A antibodies. Indeed, RNA-Seq analysis revealed that a novel Myo18A transcript is expressed in mouse ventricular myocytes, as well as in human heart. Cloning and sequencing confirmed that the cardiac isoform, termed Myo18Aγ, lacks the PDZ-containing N-terminus characteristic of Myo18Aα and incorporates alternative N- and C-termini. EGFP-tagged Myo18Aγ localized to the A-bands of sarcomeres in transfected ventricular myocytes. Furthermore, electron microscopy revealed highly disorganized sarcomeres in the hearts of *Myo18a*-deficient E10.5 embryos. In conclusion, we have identified a novel isoform of Myo18A (Myo18Aγ) which is important for cardiac sarcomere assembly and/or maintenance. Hence, both members of class XVIII myosins, Myo18A and Myo18B, are essential components of cardiac sarcomeres.

P109/B112

Aha1, a Hsp90 Co-chaperone, Is Dispensable for Hsp90a1 Function in Sarcomere Organization during Muscle Development.

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Heat shock protein 90s (Hsp90s) are molecular chaperones that play critical roles in protein folding, maturation and function. Our previous studies demonstrated that Hsp90a1 is essential for muscle development. Loss of *hsp90a1* resulted in myosin protein degradation and defective sarcomere organization in skeletal muscle of zebrafish embryos, leading to early larval lethality around 5 dpf. Hsp90s is a dimeric molecular chaperone that function in an ATP-dependent client activation cycle. The most potent stimulator of the Hsp90 ATPase activity is Aha1, a co-chaperone that binds to the Hsp90 middle domain and exerts stimulatory effect on Hsp90 ATPase activity. To determine the expression and function of Aha1 in the Hsp90 ATPase activation cycle to control the assembly of a large group of client proteins into a complex sarcomere structure in muscle cells of zebrafish embryos under normal and stress conditions. Zebrafish genome contains two *aha1* genes, namely *aha1a* and *aha1b*. We showed that under normal physiological condition, *aha1a* was predominantly expressed in heart and skeletal

muscles of the zebrafish embryo, whereas *aha1b* showed a weak expression mainly in the head region. However, under stress conditions from higher temperature incubation or *hsp90a1* knockdown, we found that both *aha1a and aha1b* expression was dramatically increased. While high temperature induced a broad upregulation of *aha1a and aha1b in* the entire embryo, knockdown of *hsp90a1*, but not *hsp90a2* upregulated *aha1a and aha1b* expression specifically in skeletal muscles. This muscle-specific upregulation was also observed in the *smyd1* mutant embryos that have disrupted sarcomere organization. To investigate whether Aha1a and Aha1b are involved in muscle development, we knocked out both genes by CRISPR in zebrafish. Loss of Aha1a or Aha1b alone had no effect on muscle development and fish survival. The expression of *hsp90a1* and *hsp90a2* was normal in both mutant embryos. However, *aha1a* expression was significantly upregulated in Aha1b mutant embryos, suggesting a compensatory response and possible functional redundancy. To completely abolish Aha1 function, we generated the *aha1a;aha1b* double mutants. The sarcomere organization revealed by anti-myosin and phalloidin staining appeared normal in the double mutant embryos, suggesting that Aha1a and Aha1b are dispensable for Hsp90a1 function in sarcomere organization under normal conditions. Future studies will be focused on characterizing the long-term effect of *aha1a;aha1b* double mutation in stress conditions from high temperature or in Hsp90a1 or Smyd1 heterozygous mutant background to assess haploinsufficiency.

P110/B113

Cardiomyocytes Seen as Active Matter: Heterogeneities and Collective Dynamic Instabilities in Sarcomere Contractions.

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Cardiac muscle contraction involves highly coordinated dynamics, from the level of the myosin motors on the length-scale of nanometers to that of the whole organ on the scale of centimeters. The molecular dynamics of myosin motors and the macroscopic motion of whole muscles are well studied. Many features of muscle contraction that are not well understood, however, emerge on the mesoscopic length scale of (half-)sarcomeres, the basic contractile units of muscles consisting of around 300 mechanically coupled myosin molecular motors. Basic theories of collective molecular motor dynamics predict emergent phenomena such as dynamic instabilities and spontaneous oscillatory motion due to non-monotonic force-velocity relations. On the next level of the hierarchy, when half-sarcomeres are coupled in series and in parallel, even richer emergent dynamics are expected. We have imaged sarcomere dynamics in individual stem-cell-derived cardiomyocytes with endogenous fluorescent labeling of z-lines introduced by CRISPR/Cas9, and have observed that mechanical competition leads to sarcomere de-coherence and complex dynamic heterogeneity. We model the observed phenomena with a dynamic myofibril model of multiple force-generating elements with non-monotonic force-velocity relations from a complex systems / active matter perspective.

P111/B114

Optimized Filopodia Formation Requires Myosin Tail Domain Cooperation.

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Filopodia are actin-filled protrusions employed by cells to interact with their environment. Filopodia formation in Amoebozoa and Metazoa requires the phylogenetically diverse MyTH4-FERM (MF) myosins DdMyo7 and Myo10, respectively. While Myo10 functions as an antiparallel dimer, DdMyo7 lacks a coiled-coil domain in its N-terminal tail region, called the proximal lever arm (PLA), raising the question of how such divergent motors perform similar functions. A series of DdMyo7 deletion mutants were tested for their ability to target to the cortex and rescue filopodia formation in the *myo7*null mutant. The tail domain consists of the PLA and two MyTH-FERM (MF) domains separated by an SH3 domain. Deletion of the PLA or the C-terminal MyTH-FERM (MF2) domain does not impact filopodia formation, however, deletion of both together does, revealing that the DdMyo7 PLA works in cooperation with the C-terminal MF domain to promote filopodia formation. An alytical ultracentrifugation studies showed that the proximal tail can dimerize, albeit weakly. The results suggest that DdMyo7 function requires partner-mediated dimerization, where an MF2 binding partner brings two myosins together at the cortex, enabling interactions between them and formation of a stable dimer. Cortical targeting of DdMyo7 is required for filopodia initiation and is controlled by head-tail autoinhibition. Interestingly, the SAH and N-terminal region of the PLA are needed to regulate the motor's autoinhibition, possibly by facilitating the proper conformation of the inactive state. The PLA is thus proposed to allow for sensitive regulation of myosin activity by preventing inappropriate activation of filopodia formation. Several Metazoan MF myosins, such as Myo7A and Myo15, also lack clear dimerization sequences but there is evidence that they are dimerized by partner binding, suggesting that this is an ancient and conserved mode of regulating MF myosin function. The results reveal that the basic principles of MF myosin-based filopodia formation are conserved but that the filopodial myosins DdMyo7 and Myo10 employ divergent mechanisms for dimerization. Supported by grants from CNRS, ANR and Ligue Contre le Cancer (AH); NIGMS, NIH (F31GM128325 to AA and R01GM122917 to MAT)

P112/B115

Relgulatory Light Chain Orientaiton on Skeletal Myosin Using a Bifunctional Spin Label.

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We performed electron paramagnetic resonance (EPR) on skeletal myosin, labeling the regulatory light chain (RLC) with a bifunctional spin label (BSL). To achieve stereoselective site-directed labeling with BSL, we engineered a pair of cysteines (i, i+4) in RLC. By exchanging BSL-labeled RLC onto myosin head (S1)/heavy meromyosin (HMM) and decorating oriented muscle fibers with BSL-RLC-S1/BSL-RLC-HMM, we obtained EPR spectra from which the angular distribution of BSL can be determined with high resolution relative to the muscle fiber axis. In our previous work (Savich et al., JGP, 2019), we exchanged BSL-RLC on intact myosin in skinned muscle fibers and observed substantial angular disorder, which varied with the RLC labeling site. In the absence of ATP (rigor), each of the two labeled helices exhibited both ordered (angular dispersion ~ 9-11 degrees) and disordered (angular dispersion > 38 degrees) populations. Using these angles to determine the orientation of the lever arm (light chain domain combined with converter subdomain), we observed that the oriented population corresponds to a lever arm that is perpendicular to the muscle fiber axis, and that addition of ATP in the absence of Ca^{2+}

(inducing relaxation) shifts the orientation to a disordered distribution. Similar orientation was observed on BSL-RLC-S1-fiber, BSL-RLC-HMM-fiber for probes located on the E helix. However, orientation of the B helix becomes completely disordered in both fragments. Addition of ATP releases decorated myosin, showing the ATP dependence of actin binding is not perturbed by labeling. Activity of labeled S1 was verified by the actin-activated NADH ATPase assay. We conclude that the lack of uniform orientation in labeled RLC, observed previously in muscle fibers by both EPR and fluorescence, is due to strain between two myosin heads. This work was supported by NIH R01AR032961 and R37AG26160 to DDT. YS was supported by NIH T32AG29796 and a University of Minnesota Interdisciplinary Doctoral Fellowship.

P113/B116

Regulation of Filamentous Actin Structural Dynamics by Myosin XV in Mechanosensory Stereocilia.

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Unconventional myosin XV (myoXV) is an actin-based motor that is essential for mechanosensory stereocilia development. MyoXV has been shown to localize to the tips of actin-based stereocilia to promote their elongation. Some mutations of *Myo15* abolish stereocilia elongation and cause deafness. However, the mechanism by which myoXV regulates stereocilia architecture remains elusive. Here, we present cryo-EM reconstructions of the wild-type and disease-causing D1647G mutant actomyosin complexes and bare filamentous actin (F-actin) at 2.75, 3.65, and 2.73Å resolution, respectively. Comparison of the wild-type actomyosin complex to F-actin alone reveals that myoXV induces apparent local structural changes at the interface. These structural changes are propagated and induce a global rearrangement of the actin subunit, which leads to a 0.2 Å reduction in the helical rise. In our naked F-actin structure, residues G47 and Q48 of the D-loop, which mediates inter-subunit contacts, are structurally flexible. Upon myoXV binding, the D-loop is partially stabilized and samples two conformations. However, the D-loop of the D1647G mutant actomyosin is completely stabilized in a single conformation. Moreover, we find that wild-type myoXV promotes, whereas the D1647G mutant inhibits, actin polymerization in *vitro*, possibly through differential modulation of the D-loop. Our results suggest myoXV could control stereocilia length by regulating F-actin structural plasticity required for accelerated actin polymerization during stereocilia elongation.

P114/B117

Unconventional Mechanisms of Actin Polymerization Drive Stereocilia Elongation in the Cochlea.

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Stereocilia are actin-based organelles that project from the apical surface of inner ear hair cells. Each hair cell assembles hundreds of stereocilia and precisely arranges them into ranks of increasing height to create a mechanosensitive hair bundle that detects sound. A para-crystalline actin core sets the architecture and mechanical strength of each stereocilium, yet how actin polymerization /

depolymerization is coordinated within these organelles remains poorly understood. An essential molecular component in the assembly mechanism is unconventional myosin 15a (MYO15A) which is necessary for establishing the height of individual stereocilia and architecture of the overall hair bundle. MYO15A is hypothesized to traffic an 'elongation complex' of proteins (WHRN, EPS8, GNAI3 and GPSM2) to the stereocilia tips; the major site of actin polymerization during elongation. How MYO15A and the elongation complex proteins collectively regulate actin polymerization is unknown. Here, we describe a missense mutation (p.D1647G) in the MYO15A ATPase (motor) domain that interferes with stereocilia growth and causes hearing loss in a mutant mouse model. Unlike previous mouse models, where molecular trafficking of MYO15A and the elongation complex were completely disrupted, MYO15A and the elongation complex were still trafficked to the stereocilia tips of p.D1647G hair cells. These data indicate that trafficking of the MYO15A-elongation complex is not sufficient for stereocilia elongation, and alludes to MYO15A having an additional function at the stereocilia tip. We hypothesized that the MYO15A ATPase domain might directly regulate actin polymerization. To test this, we purified both wild-type and p.D1647G MYO15A and studied their activity and structures bound to F-actin. Using single-particle cryo-electron microscopy, we identified structural plasticity within the actin DNAase I-binding loop ('D-loop'), that is maintained in the presence of wild type MYO15A, but suppressed by the p.D1647G mutant. To test the functional impact of this plasticity, we measured the activity of purified MYO15A using the in *vitro* pyrene-actin polymerization assay. Wild-type MYO15A strongly stimulated actin polymerization, whereas the p.D1647G MYO15A had minimal effects on polymerization kinetics. We infer that the stimulation effect by wild-type MYO15A originates from stabilizing actin-actin contacts whilst maintaining the structural plasticity required for polymerization. Our results support a new model for stereocilia growth, where MYO15A traffics proteins essential for elongation, but also directly stimulates actin polymerization at the stereocilia tip to control hair bundle architecture.

P115/B118

Src Kinases Modulate Myosin 2 Assembly.

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With actin, myosin 2 is the major driver of cell contractility. It remains indisputable that phosphorylation of the regulatory light chain at Thr18/Ser19 activates myosin 2 to induce contraction. Since it was identified in the 1980's, RLC phosphorylation has justifiably received the bulk of the attention. However, if contraction is essential throughout cell biology, and myosin 2 activity is determinant for contraction, then a model in which myosin 2 activity is largely controlled by a single mechanism should be met with skepticism. It is logical that additional mechanisms control myosin 2. Moreover, contractile events are not an on/off switch. They must be tuned to fit the cellular process: too little contractility will result in process failure; too much can literally rip cells and tissues to pieces. Therefore, cells must "sense" levels of contractility and have a mechanism to tune it. The field has described many pathways that cells use to increase contractility. We lack analogous mechanistic insight for how cells reduce or tune the level of contractility. High throughput mass spectrometry data suggests robust phosphorylation of myosin 2A at Tyr1408. This residue is in the middle of the coiled-coil tail domain, suggesting it might be important in regulating myosin 2 assembly dynamics. To explore this at the cellular level, we generated a phospho-specific antibody and an EGFP-tagged phosphoblocking mutant (myosin 2A-Y1408F). Using this antibody, we demonstrate that Tyr1408 is dynamically phosphorylated in cells. Multiple assays demonstrate expression of the phosphoblocking mutant results in severe overassembly of myosin 2 filaments. By performing an in vitro kinase screen with 42 ubiquitous or semi-ubiquitous tyrosine kinases, we observe

Tyr1408 phosphorylation by Src family kinases, including Yes1, FynA, and Src. Consistently, treatment of cells with Src inhibitors decreases myosin 2-Tyr1408 phosphorylation levels. Growth factor and adhesion signaling activate both Src kinases and myosin 2 assembly. Our data suggest a more complex model and potential negative feedback loop mediated by direct Src phosphorylation of myosin 2.

P116/B119

Morn4 Influences the Length and Dynamics of Myo3a-Associated Actin Protrusions.

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Myo3A localizes to the tips of inner ear hair cell stereocilia and plays a role in regulating stereocilia length and morphology. In addition, mutations in Myo3A are associated with non-syndromic deafness and lack of functional Myo3A causes accelerated age-dependent stereocilia degeneration. Previously we discovered that Myo3A binds a protein with four membrane occupation nexus motifs, MORN4, and the two proteins can co-localize at the tips of filopodia. In the current study, we demonstrate that MORN4 localizes to the tips of inner ear hair cell stereocilia which overlaps with the site of Myo3A localization. The presence of MORN4 enhances Myo3A localization to the tips of filopodia and increases the lengths of Myo3A-associated filopodia in COS7 cells. In addition, MORN4 alters the dynamics of Myo3A-associated filopodia by increasing the filopodia extension rate 30% without altering filopodia retraction. We investigated the dynamics of GFP-tagged Myo3A in COS7 cell filopodia in the presence and absence of MORN4 using FRAP. The half-time of Myo3A recovery at the filopodia tips is about 4-fold slower in the presence of MORN4. We found that GFP-tagged Myo3A co-expressed with MORN4 can co-localize with liposomes in vitro and cell fractionation experiments suggest Myo3A is associated with the plasma membrane. Myo3A containing the motor domain and full length tail domain can generate actin filament sliding when its tail is docked onto a motility surface using an antibody specific for MORN4 or the C-terminal tail homology domain II motif. Overall, our results suggest a model in which Myo3A generates a tip-directed force in actin protrusions and that MORN4 enhances extension and length by increasing the number of tip localized Myo3A motors.

P117/B120

Regulation of Non-muscle Myosin II Assembly by the Actin Cytoskeleton.

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Non-muscle myosin II (NMII) drives dynamic cellular processes, including cell migration, cytokinesis, and tissue morphogenesis. Upon regulatory light chain (RLC) phosphorylation, NMII self-assembles into bipolar filaments, which serve as the functional unit to slide and translocate on actin filaments to contract the F-actin network. F-actin networks in cells adopt two major architectures: linear actin filaments nucleated by formin and branched F-actin nucleated by Arp2/3. In this work, we aim to understand whether NMII assembly is regulated by different F-actin architectures. Using the fibroblast cell line 3T3 as a model system, we disassembled NMII filaments by a ROCK inhibitor Y-27632 and

subsequently washed out the drug to allow NMII filaments to reassemble. During the washout experiments, cells are treated with pharmacological inhibitors of formin or Arp2/3. The treated cells are then fixed, immunostained for NMIIA and imaged with confocal microscopy. We find that formin inhibition by SMIFH2 in conjunction with Y-27632 washout leads to a dramatic loss of NMIIA signal, whereas Arp2/3 inhibition by CK-869 does not. SMIFH2 treatment by itself also does not lead to a loss of NMIIA signal, indicating that formins play a role in regulating *de novo* NMIIA filament assembly. Using triton insoluble fractionation assays, we confirmed that NMIIA is more cytosolic and does not associate with the cytoskeleton when formin is inhibited under Y-27632 washout, suggesting that RLC phosphorylation is blocked and NMIIA remains monomeric and assembly-incompetent. Our work suggests possible feedback between formin-nucleated actin assembly and myosin filament assembly to maintain homeostasis of actomyosin contractility.

P118/B121

Quantifying Non-muscle Myosin 2 Assembly Dynamics in 4D Super-resolution.

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How cells generate sufficient contractile forces to drive a diverse array of cellular processes is an outstanding question. Non-muscle myosin 2 is a motor protein that hydrolyzes ATP to contract actin filaments, generating the majority of contractile forces in non-muscle cells. To achieve the level of contractility needed for most processes, many myosin 2 filaments are needed. For decades, the only model to explain how a sufficient number of filaments are rapidly assembled was a model in which each filament was built individually. Recent work using high-resolution imaging demonstrated an alternative model in which nascent myosin 2 filaments are amplified by an actin-dependent partitioning process. We believe this partitioning process evolved as a dominant myosin 2 assembly mechanism to rapidly drive physiological levels of contraction - we observe the phenomenon in primary mouse fibroblasts, in 3D culture, on substrates of physiological stiffnesses, and in Drosophila haematopoietic cells. We do not understand the molecular mechanism for this phenomenon. Two mechanisms have been proposed (1) Single-filament partitioning - a single myosin 2 filament (~30 monomers) is ripped in two as dynamic actin fibers separate. (2) Multi-filament partitioning - Two or more myosin 2 filaments (>>30 monomers) are separated from one another as dynamic actin fibers separate. Differentiating these mechanisms is essential for understanding contractile force generation. To do this, we are using complementary methods of molecular counting and correlative light and electron microscopy (CLEM). Using protein nanocages with fixed numbers of subunits and fluorophores, we generated a standard curve of fluorescence with super-resolution imaging. By imaging endogenously-labeled EGFP-myosin 2 with identical conditions, we can interpolate the number of myosin 2 monomers present at the time of partitioning. Our in vitro, fixed-cell, and live-cell imaging suggest that 1) in vitro single filaments are 30 monomers, 2) live-cell bipolar structures can be upward of a single filament, and 3) partitioning events appear to occur at or above that of a single filament. Collectively, this work demonstrates that we can accurately quantify dynamic macromolecular complexes, and that we can use this technique to better understand the mechanism by which cells build contractile forces.

P119/B122

Investigating the Role of *Drosophila* Myosin7a and Its Binding Partner, M7BP, in Phagocytosis and Host Defense.

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Drosophila myosin 7a (Dm7a) is an unconventional myosin required for the maintenance of the Johnston's organ (the auditory center in *Drosophila*) and the morphology of the bristle structures on the thorax. Both of these structures are formed by bundled arrays of actin. Here we show that Dm7a is involved in the process of engulfment of bacteria particles in the *Drosophila* larval hemocytes. These hemocytes have phagocytic capability and are important for the immune response during pathogen infection. Using the UAS/GAL4 system, we express GFP-Dm7a in the larval hemocytes and show that it induces the formation of filopodia in the hemocytes and Dm7a is localized in the cortical regions of the cell and in the filopodia. We imaged the uptake of fluorescently-tagged bacteria by hemocytes expressing GFP-Dm7a. When the bacteria contact the surface of the hemocyte, linear membrane ruffles appear and wrap around the bacteria. Occasionally an extended filopodia was seen to contact a bacterium and transport it back to the cell surface. We performed a phagocytosis assay to compare the ability of wildtype hemocytes versus hemocytes isolated from Dm7a mutant to engulf bacteria and found that Dm7a mutant is defective in the uptake of the bacteria, although bacteria can bind to the cell surface. We have also identified a binding partner of Dm7a using the C-terminus FERM domain as a bait in a yeast two hybrid system and named it *Myosin 7a Binding Protein* (M7BP). M7BP protein has a potential Rab-binding domain in the N-terminus. Immunoprecipitation experiments using M7BP antibody pulled down several *Drosophila* Rab proteins. RNAi knockdown of M7BP in the hemocytes affected the filopodia of the hemocytes and also compromised the ability of the hemocytes to engulf bacteria. When wildtype hemocytes are challenged with a high dose of bacteria, they utilize the process of macropinocytosis to increase the efficiency of clearing the invading pathogens. Membrane ruffles are initiated on the surface of the hemocytes that circularize to capture the bacteria and macropinocytic cups are formed which are subsequently internalized. In the RNAi knockdown lines of Dm7a and M7BP, abnormal formation of the macropinocytic cups were observed. We are exploring the functions of Dm7a, M7BP and Rab proteins in the larval hemocytes when challenged with bacteria.

P120/B123

SRNS-associated Myo1e Mutations Have Differential Effects on Myosin 1e Activity and Stability.

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Mutations in the *MYO1E* gene, encoding Myosin 1e (Myo1e), are associated with steroid-resistant nephrotic syndrome (SRNS), a progressive kidney disease that can lead to kidney failure. Myo1e is a non-muscle motor protein moving along actin filaments that is enriched in the renal glomerular epithelial cells (podocytes). Using exome sequencing, novel *MYO1E* genotypes have been found in SRNS patients but whether these Myo1e variants directly result in SRNS is not known. Here, we set out to differentiate between the pathogenic and neutral novel *MYO1E* variants identified in SRNS patients (Sadowski et al., JASN, 2015, 26(6): 1279-89). Based on the allele frequency and conservation of the amino acid residues affected by the mutations, 7 non-truncated variants (containing point mutations and frameshift

mutations that do not cause early translation termination) were selected for further protein expression and localization analysis in cultured podocytes. EGFP-tagged human Myo1e constructs containing selected mutations were delivered into immortalized mouse podocytes using adenoviral vectors. We found that Myo1e-T119I was subject to heavy ubiquitination, suggesting that this mutant may be misfolded and unstable. Accordingly, T119I was diffusely localized in the cytosol and, unlike wild type Myo1e, it was not enriched at the cell-cell contacts or co-localized with clathrin-coated vesicles in podocytes. In contrast, Myo1e-D388H mutant was similar to the wild type myosin in protein expression level and cell-cell contact localization. Surprisingly, the rate of protein exchange of Myo1e-D388H at the cell-cell contacts, as measured by Fluorescence Recovery After Photobleaching, was decreased, and the localization of Myo1e-D388H to clathrin-coated vesicles was reduced, which suggests that this mutation alters Myo1e protein interactions in podocytes. Overall, our work has verified that two *MYO1E* alleles containing mutations in the motor domain, T119I and D388H, are likely to be pathogenic. By measuring Myo1e subcellular localization and dynamics in podocytes, we showed the distinct protein features of the two Myo1e mutants. The analysis workflow used in this project can be considered for future characterization of the pathogenicity of Myo1e variants that are identified in clinical studies.

P121/B124

It Takes Two: Ubiquitylation and P97-independent Extraction of a Cargo Adaptor from a Myosin V Complex Are Required in Parallel for the Termination of Organelle Transport.

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Transport of organelles to their correct intracellular locations is essential for cellular function and homeostasis. The specificity of localization is achieved in part by molecular motors, which attach to cargoes, via cargo-specific adaptors, and actively transport them to their destinations. Our recent studies have revealed that the release of cargoes from the motor is as important as cargo attachment. Here, we show that both ubiquitylation and regulated extraction of the cargo adaptor is required to detach cargo from myosin V. In the budding yeast *S. cerevisiae*, the vacuole/lysosome is one of several cargoes moved by a myosin V motor. Early in the cell cycle, transport initiates when myosin V binds to the vacuole-specific adaptor, Vac17. Our previous work found that arrival of the vacuole at the bud cortex activates an E3 ubiquitin ligase. Vac17 is ubiquitylated and subsequently degraded by the proteasome. This was assumed to be the last step prior to degradation. Our unpublished studies reveal that a parallel pathway is essential to release the vacuole from myosin V. We found that Yck3, a Casein Kinase I isoform, and Vps41, a component of the HOPS complex, are required for the termination of vacuole transport. Moreover, while Yck3 and Vps41 are required for the degradation of Vac17, they are not required for Vac17 ubiquitylation. Instead, Yck3 and Vps41 regulate the phosphorylation of Vac17, an event required for the extraction of Vac17 from the myosin V complex. In a proteasome deficient mutant, Vac17 accumulates in puncta that are not strictly on the vacuole or with myosin V. This unique localization enabled us to discover that Yck3 and Vps41 act upstream of the proteasome to release Vac17 from the myosin V complex. Surprisingly, this extraction is not dependent on p97/Cdc48 or Vps4, AAA ATPases that extract proteins from membranes and/or protein complexes. Together, our data suggest that Yck3 and Vps41 dependent phosphorylation, concurrent with ubiquitylation, are part of a mechanism that detaches the vacuole from Myo2. These findings provide insight into how molecular motors deliver cargoes to the right place at the right time.

P122/B125

Modeling Mitochondrial Dynamics during Mitosis.

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MyosinXIX is an unconventional, mitochondria-associated motor that has been identified as a regulator of cell division. Cells depleted of MyosinXIX show two significant phenotypes: cytokinesis failure and, in cells that successfully divide, asymmetric mitochondrial partitioning from parent to daughter cells. Since the consequences of mitochondrial dynamics during mitosis are not well characterized, we developed two inductive models to address possible mechanisms behind the phenotypes previously observed. With the first model, based on energy calculations, we compared the energy required for membrane bending to the energy exerted by the actomyosin contractile ring to determine if the presence of mitochondria could interfere with cleavage furrow activity. These results suggest that mitochondria mislocalized to the cleavage plane could pose a physical impediment to cytokinesis. With the second model, based on simulations of particle movement in a cellular environment, we investigated if enhancing mitochondrial movement supported mitochondrial redistribution prior to anaphase onset by testing whether enhanced motility and/or particle size influenced particle positioning during the time frame of cellular division. Our results indicate that particle size was inversely related to final anisometry when only motility, but not fragmentation, was enhanced. Regardless of particle size, enhanced motility had the strongest effect on anisometry decrease over time and fragmentation alone had little to no effect on particle redistribution. Our results suggest that active transport is a major factor promoting symmetric particle distribution, and indicate that motor-assisted mitochondrial movement has a role in the proper partitioning of the organelle during mitosis.

P123/B126

The MyMOMA Domain of MYO19 Encodes for Distinct Miro-dependent and Miro-independent Mechanisms of Interaction with Mitochondrial Membranes.

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MYO19 interacts with mitochondria through a C-terminal membrane association domain (MyMOMA). The specific mechanisms for localization of MYO19 to mitochondria are poorly understood. Using new promiscuous biotinylation data in combination with existing affinity-capture databases, we have identified a number of putative MYO19-interacting proteins. We chose to further explore the interaction between MYO19 and the mitochondrial GTPase Miro2 by expressing mchr-Miro2 in combination with GFP-tagged fragments of the MyMOMA domain and assaying for recruitment of MYO19-GFP to mitochondria. Coexpression of MYO19$^{898\text{-}970}$-GFP with mchr-Miro2 enhanced MYO19$^{898\text{-}970}$-GFP localization to mitochondria. Mislocalizing Miro2 to filopodial tips or the cytosolic face of the nuclear envelope did not recruit MYO19$^{898\text{-}970}$-GFP to either location. To address the kinetics of the Miro2/MYO19 interaction, we used FRAP analysis and permeabilization-activated reduction in fluorescence (PARF) analysis. MyMOMA constructs containing a putative membrane insertion motif but lacking the Miro2-interacting region displayed slow exchange kinetics. MYO19$^{898\text{-}970}$-GFP, which does not include the membrane-insertion motif, displayed rapid exchange kinetics, suggesting that the MYO19 interacting with Miro2 has higher mobility than MYO19 inserted into the mitochondrial outer membrane. Mutation of well-conserved, charged residues within MYO19 or within the switch I and II

regions of Miro2 abolished the enhancement of MYO19$^{898-970}$-GFP localization in cells ectopically expressing mchr-Miro2. Additionally, expressing mutant versions of Miro2 thought to represent particular nucleotide states indicated that the enhancement of MYO19$^{898-970}$-GFP localization is dependent on Miro2 nucleotide state. Taken together, these data suggest that membrane-inserted MYO19 is part of a larger complex, and that Miro2 plays a role in integration of actin- and microtubule-based mitochondrial activities.

Tubulins and Associated Proteins 1

P124/B128

From Cytoskeletal Function to Genome Stability: Resolving the Mystery of Tubulin Isotypes in Budding Yeast (Saccharomyces Cerevisiae).

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Microtubules (MTs) are dynamic cytoskeletal filaments polymerized from tubulin, a heterodimer of alpha- and beta- subunits. Cells typically contain multiple variants, or isotypes, of alpha- and beta-tubulin. Yet how tubulin isotypes underlie MT function remains largely obscure. Their clinical importance is also revealed by human neurological and fertility disorders caused by mutations in specific isotypes. We combined genetics, cell biology and quantitative fluorescence imaging to address the question of tubulin isotypes in the tractable budding yeast model. Unlike higher eukaryotes which have several, it has just one beta (tub2) and two alpha isotypes (Tub1 & Tub3). Yet, the contribution of each alpha isotype to cellular MT function is essentially unknown. Pioneering studies using deletion or overexpression of Tub1 or Tub3 proposed they may be functionally interchangeable and suggested differences result from expression levels. However, by employing improved methods we developed otherwise isogenic cells expressing solely Tub1 or Tub3 at levels comparable to total alpha-tubulin in wildtype cells. Our results demonstrate that each isotype normally contributes ~50% of total alpha-tubulin, showing the difference between them is not strictly quantitative. Moreover, lack of either isotype in cells expressing proper tubulin levels compromises microtubule function. Strikingly, cells harboring exclusively Tub1 or Tub3 display opposite sensitivities to microtubule poisons, altered regulation of microtubule dynamics, and differential contributions to mitotic spindle function. Additionally, we used genome-wide analysis to identify genetic interactions that are common or unique between either isotype, which further revealed the roles of each isotype in specific mitotic processes. Together, our genetic, functional, and quantitative imaging data reveal previously undetermined roles for tubulin isotypes, more explicitly in spindle positioning. The yeast spindle is positioned by two distinct pathways, and our results provide mechanistic insight into how Tub3 is vital for the proper function of the early/Kar9-dependent process while Tub1 is critical for efficient positioning by the late/Dyn1-dependent mechanism. MTs are indispensable for chromosome segregation in all eukaryotes. Thus, our results suggest tubulin isotypes are key components of the mechanisms that ensure mitotic fidelity. Overall, our study uncovers novel roles for tubulin isotypes and demonstrates how isotypes allow conserved MTs to function in diverse cellular processes.

P125/B129

Loss of Microtubule Acetylation Alters Touch Evoked Swimming and Fertility in Zebrafish.

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Microtubules carry out wide range of functions in cellular transportation, motility, cell division, structural components of cilia and flagella, cellular signaling etc. Microtubule (MT) post translational modification provides diversity that is necessary to carry out these diverse functions. MT acetylation, detyrosination, mono- and polyglycylation and mono-and polyglutamylation are some of the common MT posttranslational modifications. Past studies suggested that MT acetylation could play critical role in ciliary maintenance and normal neuronal functions. Objective of this study was to understand the role of MT acetylation in zebrafish. We generated a null mutant of MT acetylation enzyme, alpha-tubulin acetyltransferase (*atat1*). These *atat1-/-* mutants had no observable morphological phenotype in the embryo and as adults. An extensive analysis of behavioral responses in these mutants revealed a reduced touch-evoked swimming response in *atat1-/-* mutant embryos. These mutant embryos did not show any alterations in their muscle fiber organization or spontaneous swimming behavior, indicating alteration in sensory elements of touch response, consistent with the mouse mutants. Even though *atat1-/-* mutants did not show any alteration in male fertility, sperm motility analysis showed increase in non-progressive motility. We also found increased MT monoglycylation in *atat1-/-* adult testes and embryo pronephric duct. To understand whether this increase in MT monoglycylation could compensate for the loss of MT acetylation in *atat1-/-* mutant, we knocked out MT monoglycylation enzyme, Tubulin tyrosine ligase-like family, member 3 (*ttll3*) in *atat1-/-* mutant background. Preliminary data showed reduced female fecundity among zygotic double mutants of *atat1* and *ttll3*, indicating potential functional compensation of MT acetylation by MT monoglycylation. Our data suggest that MT acetylation could play important role in touch response, sperm motility and female fecundity. We also show that reduced MT acetylation may be compensated for by increased MT glycylation, suggesting that these PTMs may have partially redundant functions.

P126/B130

Differential Binding Affinity of Tau Repeat Region R2 with Neuronal-specific β-tubulin Isotypes.

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Tau is a microtubule-associated intrinsically disordered protein abundantly expressed in brain and neuronal cells. The microtubule binding domain of tau located at C-terminal has four repeat regions R1, R2, R3 and R4; which binds and stabilizes the microtubule. In several neurodegenerative diseases, tau detaches from microtubules to form insoluble aggregates leading to tauopathy. Microtubules are made up of αβ tubulin heterodimeric subunits. Seven α-tubulin and nine β-tubulin isotypes have been reported in humans till date. These tubulin isotypes show variations in the residue composition mainly at C-terminal region and bind to motor proteins and anti-mitotic drugs differently. These tubulin isotypes show tissue specific expression as their relative proportion varies significantly in different type of cells. It is also known that tau binds differently to different cell lines and can either promote or demote microtubule polymerization. However, the relative binding affinity of tau to the different β-tubulin isotypes present in different cell lines is completely unknown. Therefore, we studied relative binding affinity of Tau repeat region R2 to neuronal specific tubulin isotypes βI, βIIb, and βIII using molecular modelling approach using recently reported CryoEM structure of tau repeat region R2. Our MD simulation results show a stable complex formation in between different tubulin isotypes and TauR2

which are mainly mediated by the interactions of H12 helix and C-terminal tail region of the αβ tubulin isotypes with TauR2. Our results suggest that tau shows differential binding affinity towards various β-tubulin isotypes. The order of binding affinity of TauR2 with β-tubulin isotypes is βIII> βIIb>βI. Thus, we find that TauR2 has greater affinity towards β tubulin isotypes which are abundantly expressed in neuronal cells i.e. βIII and βIIb. Our strategy can be potentially used to understand differential binding affinity of tau towards β tubulin isotypes present in other cell lines. We hope that knowledge of precise molecular origin of differential binding affinity of tau with β-tubulin isotypes present different cell types will pave the way for developing effective treatments against tau related disorders.

P127/B131

Alp7-Mto1 and Alp14 Synergize to Promote Interphase Microtubule Regrowth from the Nuclear Envelope.

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Microtubules grow not only from the centrosome but also from various noncentrosomal microtubule organizing centers, including the nuclear envelope and pre-existing microtubules. The evolutionarily conserved proteins Mto1/CDK5RAP2 and Alp14/TOG/XMAP215 have been shown to be involved in promoting microtubule nucleation. However, it has remained elusive as to how the microtubule nucleation promoting factors are specified to various noncentrosomal microtubule organizing centers, particularly the nuclear envelope, and how these proteins coordinate to organize microtubule assembly. Here, we demonstrate that in the fission yeast *Schizosaccharomyces pombe*, efficient interphase microtubule growth from the nuclear envelope requires Alp7/TACC, Alp14/ TOG/XMAP215, and Mto1/CDK5RAP2. The absence of Alp7, Alp14, or Mto1 compromises microtubule regrowth on the nuclear envelope in cells undergoing microtubule repolymerization. We further demonstrate that Alp7 and Mto1 interdependently localize to the nuclear envelope in cells without microtubules and that Alp14 localizes to the nuclear envelope in an Alp7 and Mto1 dependent manner. Tethering Mto1 to the nuclear envelope in cells lacking Alp7 partially restores microtubule number and the efficiency of microtubule generation from the nuclear envelope. Hence, our study delineates that Alp7, Alp14 and Mto1 work in concert to regulate interphase microtubule regrowth on the nuclear envelope.

P128/B132

Tubulin Polyglutamylation Controls Axonal Traffic in Hippocampal Neurons.

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Axonal transport is one of the critical cellular processes maintaining the functions of neurons over a lifetime. Microtubules as ‘tracks’ for axonal transport are subject to posttranslational modifications (PTMs), which are expected to regulate neuronal transport. Polyglutamylation is one of the most prominent PTMs in neurons and therefore a potential mechanism involved in the regulation of axonal transport. To measure axonal transport at altered levels of polyglutamylation, we have established mouse models in which we invalidated one or several enzymes involved in tubulin polyglutamylation, and isolated primary hippocampal neurons from these mice. For particularly deleterious knockout models, we used conditional knockout alleles that can be switched to knockout in the cultured neurons

by infecting with cre-recombinase-expressing lentivirus. We have observed an increase in tubulin polyglutamylation in the neurons lacking main deglutamylases. We first measured mitochondria transport in hyperglutamylation conditions, and showed that the percentage of time mitochondria spent moving is reduced by about 50% as compared to wild type. This suggests that hyperglutamylation has an inhibitory effect on mitochondria transport. We furthermore showed an increased mitochondria mobility in neurons with decreased polyglutamylation, suggesting a fine-tuning effect of polyglutamylation levels on axonal transport. To investigate the specificity of this transport regulation, we studied the transport of other axonal cargoes. Transport of lysosomes, late endosomes and BDNF vesicles, similar to mitochondria, was negatively affected by hyperglutamylation. Thus, tubulin polyglutamylation could be a general tuning mechanism for axonal transport. Considering our earlier findings, which show that hyperglutamylation induces neurodegeneration, defects in axonal transport could be one of key molecular mechanisms that induce the degeneration of neurons with hyperglutamylation.

P129/B133

In Vitro Selection, Validation & Optimization of Synthetic Single-domain Antibodies for Tau.

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We have developed a new platform for VHH screening and have designed for this purpose a fully synthetic humanized naïve Llama VHH library containing 3x10exp9 antibodies, based on a unique scaffold with random complementary determining regions (CDRs) (1). We use a combination of phage display and subsequent yeast two-hybrid (Y2H) or yeast display screening to identify antibodies against native antigens and eventually intrabodies. From this library, we have successfully selected VHH against a variety of antigens including large proteins, haptens and receptors directly selected from cell surface expression. The affinity of our VHH is similar to the affinity of antibodies selected after animal immunization. Here, we will show the selection and characterization of VHH intrabodies against Tau and phospho-Tau. Several VHH sharing the same CDR3 recognition loop and binding to the same epitope in the C-terminus of Tau were selected. We further optimized the binding affinity of one of the Tau-specific VHH using a combination of limited random mutagenesis and Y2H screening. Our results validate the use of these VHH in *in vitro* assays, but also their application as intrabodies. (1) Moutel S. et al. Elife 2016, 19:5

P130/B134

Xmap215 Interacts with F-actin in the Embryonic Neuronal Growth Cone to Promote Microtubule-F-actin Interaction.

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Microtubules (MTs) that comprise part of the cytoskeleton in neuronal growth cones provide both a driving force and a protein signaling network that are required to extend growing ends of axons to their destinations. Pioneering MTs and their plus-end resident proteins, +TIPs, play integral roles during this growth in coordination with the F-actin cytoskeleton. However, it remains unclear how MTs and F-actin

dynamics are spatially coordinated to drive accurate growth cone steering. Here, we report that the well-characterized MT polymerase, XMAP215 (also known as CKAP5), plays an important role in mediating MT-F-actin interaction within the growth cone. We have recently conducted a series of experiments, using a combination of in *vivo and in vitro* techniques, *to* more clearly identify the mechanism of interaction of XMAP215 with F-actin. While partial knockdown of XMAP215 in *Xenopus* did not affect its well-known MT polymerization function, MT organization throughout the growth cone is affected in a drastic way. Specifically, we find that MT-F-actin alignment in the growth cone periphery is impaired, raising the question of whether XMAP215 might be mediating interactions between MTs and F-actin. We demonstrate that XMAP215 regulates MT-F-actin alignment through its N-terminal TOG 1-5 domains. Additionally, we show that XMAP215 directly binds to F-actin in *vitro* and co-localizes with F-actin in the growth cone periphery. Our findings provide the first strong evidence that XMAP215 coordinates MT and F-actin interaction in *vivo*. We suggest a model in which XMAP215 regulates MT extension along F-actin bundles into the growth cone periphery and that these interactions may be important to control cytoskeletal dynamics downstream of guidance cues.

P131/B135

All Tubulins Are Not Alike: Heterodimer Dissociation Differs among Different Biological Sources; Comparison with Dimer Association.

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Tubulin, the subunit of microtubules, is a noncovalent heterodimer composed of one α and one β-tubulin monomer. Both tubulins are encoded by multiple genes (different isotypes), which are differentially expressed in different tissues and in development. Tubulin αβ dimers are found throughout the eukaryotes, and although very similar, are known to differ among organisms. We seek to investigate tubulins from different tissues and different organisms for a basic physical characteristic: heterodimer stability and monomer exchange between heterodimers. We have shown that mammalian brain tubulin heterodimers reversibly dissociate, following the mass action law. Dissociation yields native monomers that can exchange with added tubulin to form new heterodimers. Here, we compare the dissociation of tubulins from multiple sources, including from: mammalian (rat) brain, cultured human cells (HeLa cells), chicken brain, chicken erythrocytes, and the protozoan *Leishmania*. We used fluorescence-detected analytical ultracentrifugation to measure tubulin dissociation over a >1000-fold range in concentration and found that tubulin heterodimers from different biological sources differ in K_d by as much as 150-fold under the same conditions. Furthermore, when fluorescent tracer tubulins from various sources were titrated with unlabeled tubulin from a single source (rat brain tubulin), heterologous dimerization occurred, exhibiting similar affinities, in some cases binding even more strongly than with autologous tubulin. Heterodimers also associate into complexes larger than dimers, such as tetramers (dimers of dimers), and these low-level associations will be demonstrated. These results provide additional insight into the regulation of heterodimers of tubulin from different biological sources, suggesting that monomer exchange may contribute to the sorting of α- and β-tubulin monomers that associate following tubulin folding, and that dimer - dimer association may form species important for polymerization.

P132/B136

Reconstitution of the Human Gamma-tubulin Ring Complex with Recombinant Proteins.

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Microtubule formation in cells is regulated by the gamma-tubulin ring complex (gamma-TuRC), a large, multi-protein assembly found at microtubule organizing centers. The gamma-TuRC is thought to provide a polymerization template by mimicking an intermediate in the microtubule nucleation pathway. However, the composition of gamma-TuRCs isolated from native sources can vary across species, cell type, and even cell cycle stage. Further, the minimum set of proteins required for gamma-TuRC assembly and microtubule nucleating activity is not well-defined, which has limited detailed biochemical analyses of this complex. We have successfully reconstituted human gamma-TuRCs using 8 recombinant proteins overexpressed in insect cells. Recombinant gamma-TuRCs sedimented to ~30-34 S in sucrose gradients, and contained similar subcomponent stoichiometries as native human complexes, as determined by mass spectrometry. Negative stain electron microscopy revealed that reconstituted human gamma-TuRCs take on a “ring”-shaped organization with a diameter of ~30 nm, consistent with previous descriptions of native gamma-TuRCs. We developed single molecule TIRF microscopy assays to demonstrate and analyze microtubule nucleation from individual gamma-TuRCs, confirming the integrity of our recombinant material. Our work provides insight into the biochemistry of this essential complex and opens the door to mechanistic studies of gamma-TuRC function using bottom-up approaches.

P133/B137

Functional Characterization of the Echinoderm Microtubule-Associated Protein-Like (EML) Family.

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Among the most abundant microtubule associated proteins (MAPs) found in the mitotic spindle are members of the echinoderm-microtubule-associated protein-like (EML) family. This understudied MAP family is highly conserved from echinoderms (e.g. sand dollars) to humans and is known to be involved in cell division. Misregulation of EMLs has been linked to various cancers and has recently been shown to play a role in the formation of the cerebral cortex. Despite their apparent role in cell division and their connection to human diseases, it remains unknown how EMLs bind to or regulate microtubule function in cells. Using turbidity as well as TIRF microscopy assays, we characterize the effects of EML proteins on microtubule stability and dynamics. We also present data from EML2 knock-out cells, which show abnormal growth and morphology. Our research provides new insights into the function of various members of the EML protein family.

P134/B138

Conformational Diversity of Dynactin Sidearm.

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Dynactin is a huge protein complex, which interacts with various microtubule binding proteins and vesicle binding proteins. It regulates the motility of dynein and recent cryo-EM studies revealed its averaged structure. However, our knowledge on the structure of sidearm in dynactin complex is still limited probably because of its flexibility. What conformation the sidearm adopts is an especially important question because it takes part in interaction with dynein, microtubules, and plus-end binding

proteins. Here, we report various conformations of dynactin sidearm observed by electron microscopy. We found that a basal part of sidearm (shoulder domain) was either bound to or released from the Arp1 rod and the ratio of these forms was dependent on the ionic strength. Furthermore, the dynein-binding domain in the sidearm (coiled-coil 1) adopted either a folded or an extended form. Our results indicate that dynactin complex has several intramolecular interaction sites, which enables dynactin to adopt various conformations. These features must be a result of a remarkably complicated submolecular architecture of dynactin complex and be important for dynactin to work as a versatile regulator of dynein motility in the cell.

P135/B139

The Roles of Two γ-tubulin-associated Proteins in Embryos and Differentiated Cells in *Caenorhabditis Elegans*.

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The γ-tubulin complex (γTuC) is a major nucleation factor of microtubules (MTs), accumulating to microtubule-organizing centers (MTOCs), such as centrosomes in animal cells. There are two types of γTuCs; the γ-tubulin small complex (γTuSC) composed of the γ-tubulin and GCP2 and GCP3, and the γ-tubulin ring complex (γTuRC) assembled by several γTuSCs and other proteins including GCP4-6 and MOZART1. In *Caenorhabditis elegans,* GCP-4-6 are not conserved, thus, the composition and regulatory mechanisms of the γTuC are implicated to be different from other organisms. As γ-tubulin-associated proteins, we biochemically identified two *C. elegans* proteins, GTAP-1 and -2. Yeast two-hybrid assays indicated that GTAP-1 and-2 interact with γ-tubulin and GIP-2/GCP2. Live-imaging microscopy showed that GTAP-1 and -2 localized at the PCM throughout the cell cycle in a γ-tubulin-dependent manner. The RNAi knockdown and mutations of GTAP-1 and -2 resulted in reduction of γ-tubulin proteins at centrosomes, but embryonic development was largely unaffected. However, the *gtap-1* mutant showed abnormal morphology of the germline cells and the brood size was significantly reduced. In these animals, the organization of MTs and cell membrane of the syncytial germline was disrupted, which might be caused by the failure of γTuC localization at the germ cell membrane. On the other hand, the *gtap-2* mutant showed no abnormality in the germline. These data suggest that GTAP-1 may be involved in the γTuC targeting to the germline cell membrane.

P136/B140

The Role of Alpha-tubulin Acetylation in *Drosophila*Oogenesis.

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During *Drosophila* oogenesis, microtubules are known to play several key roles including oocyte specification and differentiation, the transport of materials into the oocyte from neighboring cells, and specifying the axis of the oocyte for embryonic development. The diverse functions of microtubules are regulated by the post-translational modifications (PTMs) of the microtubule subunits, alpha- and beta-tubulin, which are subjected to a wide array of PTMs. One such PTM is acetylation of the conserved K40 residue of alpha-tubulin. Alpha-tubulin K40 acetylation has been reported to be enriched on stable microtubules where it is suggested to confer increased flexibility to microtubules and promote resistance to breakage from mechanical forces. K40 acetylation is facilitated by an alpha-tubulin

acetyltransferase, MEC17, which is counterbalanced by the activity of the major tubulin deacetylase, HDAC6. To study alpha-tubulin acetylation in oogenesis, we focused on a *Drosophila* gonad-specific Mec17 (Mec17g) and generated a knockout fly with the Mec17g locus removed (Mec17g KO). Mec17g KO flies exhibited age-dependent defects in fertility and egg formation. The defect in egg formation could be rescued by the somatic follicle cell-specific expression of either Mec17g or alpha-tubulin K40Q (an acetylated alpha-tubulin mimic), demonstrating the requirement of alpha-tubulin K40 acetylation in follicle cells for proper egg development. An alysis of Mec17g KO follicle cells by electron microscopy revealed the absence of stacked endoplasmic reticulum (ER) sheets—ER structures which have been hypothesized to permit maximal protein synthesis in professional secretory cells—suggesting a role for Mec17 in the formation and/or maintenance of stacked ER sheets.

P137/B141

Microtubule Acetylation Is Required for Mechanosensation in *Drosophila*.

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At the cellular level, α-tubulin acetylation alters the structure of microtubules to render them mechanically resistant to compressive forces. How this biochemical property of microtubule acetylation relates to mechanosensation remains unknown, although prior studies have shown that microtubule acetylation influences touch perception. Here, we identify the major *Drosophila* α-tubulin acetylase (dTAT) and show that it plays key roles in several forms of mechanosensation. dTAT is highly expressed in the larval peripheral nervous system (PNS), but it is largely dispensable for neuronal morphogenesis. Mutation of the acetylase gene or the K40 acetylation site in α-tubulin impairs mechanical sensitivity in sensory neurons and behavioral responses to gentle touch, harsh touch, gravity, and vibration stimuli, but not noxious thermal stimulus. Finally, we show that dTAT is required for mechanically induced activation of NOMPC, a microtubule-associated transient receptor potential channel, and functions to maintain integrity of the microtubule cytoskeleton in response to mechanical stimulation.

P138/B142

Understanding the Role of Yeast Map She1 in Dynein-Mediated Spindle Positioning.

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During cell division in budding yeast, cortically anchored dynein motors position the mitotic spindle within the bud neck, a narrow constriction between the mother and daughter cells. Dynein is assisted by numerous factors to perform this function, including the microtubule-associated protein (MAP) She1, which is a potent inhibitor of dynein motility in *vitro*. We previously found that She1 supports dynein-mediated spindle orientation by promoting the ability of dynein to move the spindle across the bud neck, raising the possibility that She1 affects this process by supporting nuclear transit into the narrow constriction of the neck. Here we set out to assess the role of She1 in dynein-mediated nuclear and spindle positioning, and the various factors that govern this process. We find that although She1 can promote a persistent force generating state of dynein, loss of She1 had no discernible effect on the success rate for dynein-mediated nuclear migration into the narrow bud neck. Close inspection of dynein-mediated spindle movements revealed that loss of She1 does not compromise spindle neck crossing directly, but increases the duration of time the spindle spends in close proximity of the mother and daughter cell cortices. Our data indicate this is likely a consequence of the increased dynein activity

also seen in cells lacking She1, suggesting that by attenuating dynein activity, She1 maintains the spindle within close proximity of the bud neck, the site of anaphase onset, and the future site of cell division. Although She1 has previously been shown to affect dynein-dynactin interaction in cells, we find that the ability of She1 to restrict dynein-mediated spindle movements to the bud neck region likely occurs in a dynactin-independent manner. Moreover, observations of cells expressing a mutant tubulin or a mutant dynein, which both exhibit reduced She1 binding, revealed an increased cortical proximity phenotype similar to that observed in *she1Δ* cells. Taken together, our data support a model whereby She1 attenuates dynein activity through direct interactions with the dynein MTBD and the microtubule, which ensures appropriate positioning of the mitotic spindle at the future site of cytokinesis.

P139/B143

Development of an Optogenetic Tool to Reversibly Control Microtubule Acetylation.

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Acetylation of α-tubulin at lysine-40 is a highly conserved post-translational modification, which is reported to stabilize microtubule against bending stresses. Microtubule acetylation is exclusively catalysed by α-acetyltransferase-1 (ATAT1), whose only known substrate is α-tubulin. Despite being implicated in a wide array of physiological phenomena including ciliary function, cell motility and mechanosensation, the precise role of microtubule acetylation in these events is not well understood, due to a limited understanding of ATAT1 regulation and lack of tools to specifically control microtubule acetylation. Here we report development of an optogenetic tool to control microtubule acetylation in live cells in a reversible manner. We observed that ATAT1 shuttles between nucleus and cytoplasm but maintains a predominantly cytoplasmic localization by active nuclear export in a Crm1-dependent manner. Unlike wild-type ATAT1, exogenous expression of nuclear localized ATAT1 catalytic domain did not significantly increase microtubule acetylation, suggesting that nuclear export may play a role in regulating ATAT1 access to microtubules and thus regulate acetylation levels. We identified a conserved Leptomycin-B sensitive nuclear export sequence in the disordered carboxy-terminal region, which is critical for nuclear export but not catalytic activity of ATAT1. Based on these findings, we hypothesized that inducible translocation of ATAT1 catalytic domain from nucleus to cytoplasm should allow us to control microtubule acetylation levels. We modified the previously described Light-inducible nuclear export system (LEXY) to reduce its dark-state activity and tethered the catalytic domain of ATAT1 to its carboxy-terminus. LEXY-ATAT1 was predominantly nuclear in dark and was rapidly shuttled to the cytoplasm upon blue light stimulation in a reversible manner. A lit mutant of LEXY(lit)-ATAT1 induced around two-fold increase in microtubule acetylation levels in HeLa cells, as compared to a non-transfected cells or cells expressing a dark mutant of LEXY(dark)-ATAT1. HeLa cells expressing LEXY-ATAT1 exposed to light for 4 hours showed a similar two-fold increase in microtubule acetylation compared to those kept in dark, validating the functionality of the tool. Thus, we have developed a powerful tool to control microtubule acetylation that will provide various applications exploring cell motility and ciliary functions.

P140/B144

Systems Biology Identifies Gleevec as a Specific Inhibitor of CLIP-170S, a Novel +Tip Isoform, Which Causes Taxane Resistance in Cancer Cells and Patients by Obstructing the Microtubule Pore.

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Taxanes are widely used in the treatment of solid tumor patients including gastric cancer (GC). Post-hoc analysis of the clinical trial that led to docetaxel approval in GC, revealed that patients with diffuse histological subtype were intrinsically resistant to taxanes. As yet, the molecular basis of clinical drug resistance remains poorly elucidated. Using a panel of GC cell lines, we identified a subset with intrinsic taxane resistance due to impaired drug-target engagement, in the absence of tubulin mutations or decreased drug accumulation. We discovered a novel, short variant of the microtubule (MT) +TIP binding protein CLIP-170, hereafter CLIP-170S, which was preferentially expressed in resistant cells. Mass-spec proteomics and 5'RACE showed that CLIP-170S lacked the first 150 amino acids, thus, missing the Cap-Gly domain required for +TIP localization. Microscopy of endogenous or exogenous proteins revealed that CLIP-170S was mislocalized from +TIP to the MT lattice in contrast to the canonical CLIP-170. Stable CLIP-170S knock down (KD) entirely reversed taxane-resistance (~300 fold), directly establishing CLIP-170S as the cause of taxane resistance. Quantitation of Flutax-2 (fluorescently labeled taxane) binding kinetics by live-cell imaging of native cytoskeletons in sensitive and resistant cells, showed that Flutax-2 dissociated faster from MTs in CLIP-170S-expressing resistant cells due to slower association rate. CLIP-170S-KD fully restored Flutax-2 binding to MTs, indicating that CLIP-170S impedes taxane-MT interaction. As taxane binding to MT lumen requires entry via the MT pore, we used chemical probes binding at the outer-only (hexaflutax) or luminal (cyclostreptin) pore sites and showed reduced binding of both compounds to resistant cell cytoskeletons. In contrast, CLIP-170S had no effect on peluroside whose MT binding does not require access through the pore. Together, these data indicate that CLIP-170S obstructs the MT pore, preventing drug access to the MT lumen and causing taxane resistance. Clinically, we found CLIP-170S to be expressed in ~60% of GC patient tumors and that its expression was significantly associated with resistance to cabazitaxel monotherapy. Computational analyses of RNAseq data from sensitive and resistant cells predicted Gleevec (Imatinib) as a drug that could overcome taxane resistance. Indeed, we showed that Gleevec reversed taxane resistance by specific depletion of CLIP-170S protein. Taken together, these data reveal an entirely novel mechanism of taxane resistance via obstruction of the MT pore by the previously unrecognized CLIP-170S. We further found CLIP-170S to be highly prevalent in patient tumors and identified Gleevec as the first specific inhibitor of CLIP-170S.

P141/B145

Systematic Characterization of a Large Number of Microtubule-Associated Proteins Using Purification-free Tirf-reconstitution Assays.

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The microtubule cytoskeleton is complex filamentous network involved in diverse functions such as cell division, cell shape, neuronal differentiation, ciliary beating. Strict regulation of microtubule functions is therefore of high importance for the integrity of the healthy cell, and perturbations are often linked to diseases like cancer, ciliopathies and neurodegeneration.In a cellular context, microtubule properties can be controlled by two major mechanisms: by the expression of alternative tubulin genes and post-translational modifications of tubulin, or/and via the interaction of microtubules with a large variety of microtubule-associated proteins (MAPs). Our knowledge of such interactors has been continuously enriched over the past decades, but up to this date no systematic studies exist that aim to describe and categorize these proteins according to their binding mechanisms and structural effects on microtubules. In the present work, we have developed an assay for rapid and systematic analysis of around 40 MAPs, using cleared lysates of cultured human cells in which we overexpress the MAPs of interest. The dynamic behaviour of growing microtubule in presence of different MAPs were monitored over time using total internal reflection fluorescence (TIRF) microscopy. This allows us to study the behaviour of large number of MAPs in a situation close to their natural environment, but eliminating cellular complexity coming from different organelles and crammed cytoskeleton filaments inside the confined intracellular space. Indeed, most MAPs were nicely soluble in our extract approach, while purification often led to protein precipitation. Our novel approach allowed us to define several novel proteins as bona-fide MAPs. We show that previously uncharacterized MAPs have strikingly different effects on microtubule polymerization and microtubule structure, thus creating a variety of distinct microtubule arrays. Also, our cell free TIRF assays provide the evidence for a direct role of many MAPs in the coordination of microtubule and actin cytoskeleton arrays. Moreover, MAPs such as Tau and MACF1 clearly show a preference for PTM-rich microtubules. Our experiments will allow for a better mechanistic understanding of how MAPs and tubulin heterogeneity together control cytoskeleton functions.

P142/B146

Microtubule Glycylation Promotes Attachment of Basal Bodies to the Cell Cortex.

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Motile cilia generate directed hydrodynamic flow that is important for the motility of cells and extracellular fluids. To maximize the use of forces generated by beating motile cilia, cells organize and orient hundreds of motile cilia into polarized arrays. Basal bodies (BBs) nucleate and position motile cilia at the cell cortex. Cytoplasmic BB-associated microtubules are conserved structures that extend from BBs and reinforce BB and cilia positioning. Using the ciliate, *Tetrahymena thermophila*, we show that BB-appendage microtubules attach to the cell cortex through several independent orientations. BB-

appendage microtubules are assembled shortly after new BB assembly and finish their elongation prior to when BBs nucleate cilia. These BB-appendage microtubules are specifically marked with post translational modifications of tubulin, including glycylation. Mutations that prevent glycylation shorten BB-appendage microtubules and disrupt BB organization, orientation and cortical attachment. Microtubule glycylation is specifically important for maintaining the position of BBs against the forces from high-frequency ciliary beating. Consistent with the attachment of BB-appendage microtubules to the cell cortex for BB positioning, mutations that disrupt the cellular cortical cytoskeleton similarly disrupt the cortical attachment and positioning of BBs. In summary, BB-appendage microtubules promote the organization of ciliary arrays through attachment to the cell cortex.

Microtubule Dynamics and its Regulation 1

P143/B147

Domain an alysis of PRC1/Ase1 Reveals Distinct Interactions with Microtubule Surface That Regulate an aphase Spindle Elongation.

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Proper chromosome segregation during mitosis requires structural rearrangement of the microtubule network. Microtubule crosslinkers such as kinesin-5 and PRC1/Ase1 are key to this process, promoting the formation and elongation of a bipolar spindle. During mitosis Ase1 localizes to antiparallel overlapping microtubules, but how Ase1 activity is regulated to promote the progression of structural re-arrangements in the spindle is poorly understood. We investigated the domains of Ase1 that are required for spindle function and discovered important roles for the microtubule-binding spectrin-homology domain and the disordered carboxy-terminal domain. We hypothesize that Ase1 loading and diffusion along the microtubule surface is mediated by phospho-regulated interactions between these Ase1 domains and the negatively-charged carboxy-terminal tails of the tubulin subunits. In support of our hypothesis, we find that a partial truncation of the Ase1 carboxy-terminal domain reduces anaphase Ase1 loading on to the spindle and disrupts Ase1's ability to dynamically slow anaphase B elongation rates. Complementing these experiments, deletion of the tubulin carboxy-terminal tails results in increased pre-anaphase Ase1 spindle localization. However, this increased localization is dependent on Ase1's carboxy-terminal domain, as truncations of both the Ase1 carboxy-terminal domain and the tubulin carboxy-terminal tails result in decreased spindle localization. Furthermore, the spectrin-homology domain contains three positively-charged basic amino acids predicted to interact with the β-tubulin tail, and mutating these three amino acids to alanine impacts Ase1 function in a distinct manner from carboxy-terminal domain truncations. These mutations decrease Ase1's spindle localization and consistently reduce Ase1's braking ability throughout anaphase B spindle elongation. Faster spindle elongation impairs spindle stability and may impact genome integrity. Overall, our results suggest Ase1 uses multiple binding interfaces with the microtubule to regulate distinct stages of spindle formation and function, and that this mechanism involves regulated interactions with the tubulin carboxy-terminal tails to provide a dynamic braking system during anaphase.

P144/B148

Katanin Grips the Beta-tubulin Tail through an Electropositive Double Spiral to Sever Microtubules.

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The AAA ATPase katanin severs microtubules. It is critical in cell division, centriole biogenesis and neuronal morphogenesis and its mutation causes microcephaly. The microtubule templates katanin hexamerization and activates its ATPase. The structural basis for these activities and how they lead to severing is unknown. Here, we show that beta-tubulin tails are necessary and sufficient for severing. Cryo-EM structures reveal the essential tubulin tail glutamates gripped by a double spiral of electropositive loops lining the katanin central pore. Each spiral couples allosterically to the ATPase and binds alternating, successive substrate residues, with consecutive residues coordinated by adjacent protomers. This tightly couples tail binding, hexamerization and ATPase activation. Hexamer structures in different states suggest an ATPase-driven, ratchet-like translocation of the tubulin tail through the pore. An essential disordered region outside the AAA core anchors katanin to the microtubule while the AAA motor exerts the forces that extract tubulin dimers and sever the microtubule.

P145/B149

Unc-45a Is a Microtubule Severing Protein.

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UNC-45A, is a highly conserved member of the UCS (UNC45A/CRO1/SHE4P) protein family [1-3]. We have shown that it plays an important role in regulating cytoskeletal-associated functions including cytokinesis[4], exocytosis[5], cell motility[4], and neuronal development[6]. Most of what we currently know about UNC-45A pertains to its role as a regulator of the actomyosin system. Emerging studies from both our and other laboratories support a role of UNC-45A outside of actomyosin regulation. This includes studies showing that UNC-45A: regulates gene transcription, co-fractionates with gamma tubulin and regulates centrosomal positioning and is found in the same subcellular fractions where MT-associated proteins are. Recent studies from our laboratories indicate that UNC-45A functions as a Microtubule Associated Protein (MAP) with MT destabilizing properties[7, 8]. Specifically: ***a)*** using TIRF microscopy, we found that UNC-45A directly binds to MTs in the absence of any additional cellular cofactors and acts as an ATP-independent MT destabilizer, ***b)*** UNC-45A destabilizes MTs in *vivo in* absence of its myosin binding domain and in even in presence of the myosin II inhibitor blebbistatin, and ***c)*** UNC-45A is overexpressed in paclitaxel resistant cancer cells. Mechanistically, UNC-45A's MT destabilizing activity is consistent with MT bending and severing. Inhibition of UNC-45A causes mitotic defects consistent with excessive MT stability and restores cancer cells' sensitivity to paclitaxel. These findings reveal novel and significant roles for UNC-45A in regulation of cytoskeletal dynamics, broadening our understanding of the basic mechanisms regulating MT stability and human cancer susceptibility to paclitaxel. ***References*** 1. Hoppe, T., et al., Cell, 2004. **118**(3): p. 337-49. 2. Price, M.G., et al., J Cell Sci, 2002. **115**(Pt 21): p. 4013-23. 3. Barral, J.M., et al., Science, 2002. **295**(5555): p. 669-71. 4. Bazzaro, M., et al., Am J Pathol, 2007. **171**(5): p. 1640-9. 5. Iizuka, Y., et al., J Immunol, 2015. **195**(10): p. 4760-70. 6. Iizuka, Y., et al., Mol Biol Cell, 2017. **28**(10): p. 1337-1346. 7. Mooneyham, A., et al., Mol Cancer Res, 2019. **17**(2): p. 370-383. 8. Habicht, J., et al., Cancer Biol Ther, 2019: p. 1-10.

P146/B150

XMAP215 Promotes Microtubule Catastrophe by Promoting Ragged Microtubule End Structures.

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The microtubule cytoskeleton is essential for a variety of cellular processes, including intracellular transport, cell motility, and cell division. Dynamic remodeling of the microtubule network is facilitated by microtubule dynamic instability, the stochastic switching of individual microtubules between phases of growth and shrinkage. A growing microtubule maintains a stabilizing cap of GTP-tubulin at its end; if this GTP-cap is lost, the microtubule undergoes 'catastrophe' and switches into a phase of shrinkage. The stabilizing GTP-cap can be visualized through microtubule end-localization of EB proteins, known to recognize the nucleotide state of tubulin within the microtubule. An increase in microtubule growth rate, when achieved by increasing tubulin concentration in *vitro*, correlates with an increase in the size of the 'EB comets', indicating that the GTP-cap size scales with the microtubule growth rate. At the same time, increasing tubulin concentration results in a lower catastrophe frequency, consistent with the hypothesis that catastrophe frequency negatively scales with the GTP-cap size. However, microtubule growth rates achieved with tubulin alone in *vitro* are an order of magnitude lower than those typically observed in cells. Fast microtubule growth rates can be reconstituted in *vitro* through synergistic effects of EB1 and XMAP215, a well-studied microtubule polymerase. How this combination of proteins impacts microtubule catastrophe frequency and the GTP-cap size is unknown. Here, we observe that fast microtubule growth rate achieved using XMAP215 and EB1 in *vitro* correlates with increased GTP-cap size. Interestingly, we show that the addition of XMAP215 simultaneously increases both microtubule growth rate and catastrophe frequency, contrary to what is observed when microtubule growth rates are increased using tubulin alone. Using total internal reflection fluorescence with structured illumination microscopy and negative stain electron microscopy, we find that microtubules grown with XMAP215 display a higher frequency of ragged microtubule end structures, in which a subset of protofilaments are growing faster than others. The structural destabilization of growing microtubule ends correlates with the observed increase in microtubule catastrophe. Our results underscore the role of the dynamic evolution of microtubule end structure in dictating overall microtubule stability.

P147/B151

The Concerted Actions of Tip1/clip-170, Klp5/kinesin-8, and Alp14/xmap215 Regulate Microtubule Catastrophe at the Cell End.

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Spatial regulation of microtubule catastrophe is important for controlling microtubule length and consequently contributes to the proper establishment of cell polarity and cell growth. The +TIP proteins including Tip1/ CLIP-170, Klp5/Kinesin-8, and Alp14/XMAP215 reside at microtubule plus ends to regulate microtubule dynamics. In the fission yeast Schizosaccharomyces pombe, Tip1 and Alp14 serve as microtubule stabilizing factors while Klp5 functions oppositely as a catastrophe promoting factor. Despite that Tip1 has been shown to play a key role in restricting microtubule catastrophe to the cell end, how Tip1 fulfills the role remains to be determined. Employing live-cell microscopy, we showed that the absence of Tip1 impairs the localization of both Klp5 and Alp14 at microtubule plus ends but the absence of Klp5 prolongs the residence time of Tip1 at microtubule plus ends. We further revealed that Klp5 accumulates behind Tip1 at microtubule plus ends in a Tip1 dependent manner. In addition, artificially tethering Klp5 to microtubule plus ends promotes premature microtubule catastrophe while

tethering Alp14 to microtubule plus ends in the cells lacking Tip1 rescues the phenotype of short microtubules. These findings establish that Tip1 restricts microtubule catastrophe to the cell end likely by spatially restricting the microtubule catastrophe activity of Klp5 and stabilizing Alp14 at microtubule plus ends. Thus, the work demonstrates the orchestration of Tip1, Alp14, and Klp5 in ensuring microtubule catastrophe at the cell end.

P148/B152

Spastin Is a Dual-function Enzyme That Severs Microtubules and Promotes Their Regrowth to Increase the Number and Mass of Microtubules.

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Microtubules are dynamic cytoskeletal filaments that play central roles in eukaryotic cell division, migration and tissue development. One important family of microtubule regulators comprises the severases—spastin, katanin and fidgetin that use ATP-hydrolysis to cut microtubules into short fragments. Whereas one might anticipate severing as a destructive process for microtubules network, previous in *vivo* studies have demonstrated that inhibiting these enzymes contradictorily decreases microtubule number and mass in cells, suggesting microtubule severases may possess nucleation-like activity. To resolve this paradox, we reconstituted a dynamic microtubule severing system in *vitro and* found that *Drosophila* spastin is a dual function microtubule regulator. Besides the canonical severing activity, spastin slows down microtubule shrinkage and increases rescue through an ATP-independent mechanism. Accumulation of spastin on the shrinking microtubule ends is observed in optical microscopy and may underlie its effect on microtubule shrinkage and rescue. By increasing rescue and slowing down shrinkage, spastin switches the microtubules into their unbounded growth regime and promotes the regrowth of severed microtubule fragments. This leads to the increase in microtubule number and mass, accounting for the in *vivo* phenotype of spastin. An extended dynamic instability model demonstrates that spastin's effect on microtubule dynamics is essential for increasing microtubule mass and number. The mathematical model further reveals that severing activity and microtubule growth rate are the key factors for determining the steady-state length distribution, and the functional relation between severing rate and steady-state mean length closely resembles a power law. These results provide a quantitative basis of how severases can modulate microtubule network in the complex cellular environment.

P149/B153

Brownian Dynamics Modeling of Microtubule Dynamics and Force Generation Offers a Role for the Flared Morphology of Growing Microtubule Tips.

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Microtubules (MTs) are essential cytoskeletal polymers in all eukaryotic cells. Thanks to their dynamic instability, MTs efficiently search and capture chromosomes in mitosis. Upon association with chromosome-bound kinetochore proteins, MT tips produce pulling and pushing forces that aid in the accurate segregation of sister chromatids. Understanding of the mechanisms that underlie these

processes has been limited by our lack of knowledge of MT tip structure and the conformational changes responsible for MT switches between assembly and disassembly. In contrast to most previous models, our recent descriptions of MT tips by electron tomography in vivo (six species) and in vitro (under several experimental conditions) found that the ends of these dynamic polymers display flaring protofilaments in both growing and shrinking states (McIntosh et al., 2018). Curved protofilament morphology at MTs ends may have profound implications for MT dynamics, for mechanical force generation, and for the regulation of both processes by associated proteins. However, existing models for MT dynamics have not yet explicitly considered this structural feature, nor have they employed data from MT force generation experiments. Here we use a Brownian dynamics method to construct and systematically analyze a comprehensive model for MT dynamics and force production. We demonstrate that a description of MT assembly and disassembly with flared ends can be achieved under a range of conditions, using simple tubulin lateral interaction energy potentials. However, force generation experiments put constraints on model parameters, pointing to the presence of a high and steep activation energy barrier in the lateral tubulin interaction energy potential. When properly constrained, the model describes the development of large pulling forces by shortening MTs and considerable pushing forces by growing MTs. Moreover, the flared morphology of growing MT tips enables sustained assisting forces, mediated via a circular kinetochore coupler. A load-dependent acceleration of MT growth rates provides an explanation for the long-standing problem of synchronizing the assembly and disassembly of MTs connected to opposite spindle poles during metaphase chromosome oscillations.

P150/B154

A Model for Ftsz Treadmilling and Nucleation Based on R->T State Conformations and Gtp Hydrolysis, and Potential Application to Assembly of Microtubules.

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(A) Bacterial tubulin homolog FtsZ assembles single-stranded protofilaments (PFs) that treadmill, adding subunits at the bottom and losing them at the top. We have designed a Monte Carlo (Gillespie) model based on a transition from an R(elaxed) state, with low affinity for PF assembly, to a T(ense) state with high affinity. FtsZ monomers are mostly R, while subunits in a PF switch to T. GTP hydrolysis weakens the longitudinal affinity, but if the GDP is in the middle of the PF the T-state interfaces remain strong enough to avoid fragmentation. However, the top subunit has no subunit above it to enforce its T state, and a GDP below allows it to switch to R and rapidly dissociate. Treadmilling is powered by a GTP->GDP gradient, as GTP subunits associate at the bottom and hydrolase stochastically. Although the PF top is mostly GDP, there is a finite probability of having a GTP, which would initiate a GTP cap on top. Our model uses kinetics suggested by A. Wegner (JMB 108:139, 1976) to erode this cap and permit treadmilling to continue. The model matches treadmilling velocity, GTP turnover and PF length distribution. **(B)** Our model also provides a natural mechanism for nucleation. Monomeric FtsZ exists in a ~5000:1 R:T equilibrium, where the small fraction of T monomers serve as nuclei. R monomers bind to the bottom of a T monomer with the same kinetics as to a PF bottom. The model matches published nucleation curves. **(C)** Recently McIntosh et al (JCB 217:2691) presented cryoEM evidence that MTs flair into single PFs at both growing and shrinking ends. This suggests that subunits add by forming a single longitudinal bond at the end of a PF, a major departure from lattice models that invoke lateral bonds and cozy corners. Tubulin is known to have R and T-state conformations similar to FtsZ. We suggest that the R->T transition may allow for cooperative MT assembly at the level of single flared PFs. Lateral bonds are essential for making the MT wall, but are only formed several subunits below the PF tip, where

subunits add by forming a single longitudinal bond. This mechanism has been detailed in Biophys J 116:1-6.

P151/B155

CENP-E/kinesin-7 and KIF4A/kinesin-4 Sequentially Drive Microtubule Flux in Human Mitotic Spindles.

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Mitotic spindle microtubules (MTs) in metazoans undergo poleward flux, which has been proposed to improve chromosome segregation fidelity. Despite that its presence have been observed for decades, the molecular and functional basis underlying spindle MT flux remains controversial. Here, we re-investigated several known candidates required for flux (kinesin-5/EG5, kinesin-13/KIF2A, CLASP1+2), while testing additional potential players (kinesin-12/KIF15, kinesin-7/CENP-E, kinesin-10/hKID, kinesin-4/KIF4A). We identified KIF4A as a particularly strong driver of MT-flux during late prometaphase/metaphase. This depended on its motor activity and localization on chromosomes. In agreement, expelling the chromatin from the spindle in cells undergoing mitosis with unreplicated genomes (MUGs), led to a strong reduction in flux rates, suggesting a role for chromosome arms in MT-flux generation. To examine how kinetochore (KT)-MT end-on attachments impact MT-flux, we measured flux rates in cells depleted of NDC80/HEC1. Intriguingly, MTs in these cells fluxed faster, indicating that KT-MT end-on attachments work as flux brakes, consistent with a coupled-spindle model in which fluxing non-KT MTs transmit force to KT MTs via MT-crosslinking. Testing this, we found that depletion of the MT crosslinking proteins NuMA and kinesin-14/HSET, but not PRC1, reduced MT-flux rates only when end-on attachments were established. Moreover, this resulted in asynchronous MT-flux within different spindle MT subpopulations. Thus, MT-crosslinking molecules ensure the equal distribution of MT-flux-dependent forces across the mitotic spindle. Most surprisingly, CENP-E inhibition strongly reduced MT-flux, specifically in NDC80/HEC1 depleted cells, suggesting a role in MT-flux generation before the establishment of end-on KT-MT attachments. Taken together, these data support a model in which MT-flux in early mitosis is largely driven by CENP-E, which localizes at KTs and interpolar MTs throughout mitosis. Upon chromosome congression, most of CENP-E is stripped from KTs and KIF4A cannot move the chromosomes any further due to equivalent opposite forces. Consequently, KIF4A causes chromatin-interacting MTs to flux towards the poles. KT MTs flux due to spindle MTs coupling via MT cross-linkers. Spindle length is maintained by the polymerizing and depolymerizing activities of CLASPs and KIF2A, respectively.

P152/B156

Dynamics of Microtubule Doublet Assembly.

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Microtubule doublets (MTDs) are structures found in cilia serving as scaffolding elements and double-track railways for intraflagellar transport trains. These MTDs are formed by a complete A-microtubule (A-MT) composed of 13 protofilaments and an incomplete B-microtubule (B-MT) of 10 protofilaments.

Despite the fundamental role of MTDs, the molecular mechanism governing their formation is unknown. We used a cell-free assay to demonstrate a crucial inhibitory role of the carboxyl-terminal (C-terminal) tail of tubulin in MTD assembly. Removal of the C-terminal tail of an assembled A-microtubule allowed for the nucleation of a B-microtubule on its surface. C-terminal tails of only one A-microtubule protofilament inhibited this side-to-surface tubulin interaction, which would be overcome in vivo with binding protein partners. Cryo-ET of these reconstituted MTDs showed structural similarity to the ciliary MTDs with a typical triangular outer junction.Live imaging of MTD assembly from fluorescently-labeled tubulin revealed that the B-MT nucleate on the surface of the A-MT and showed isotropic elongation in the presence of GMPCPP, a non-hydrolyzable analogue of GTP. By contrast, in the presence of GTP B-MTs' tips exhibited dynamic instability similar to normal dynamic microtubule behavior.Collectively, these data shed light on the crucial role of tubulin C-terminal tails in regulating MTDs and dynamics of B-MT nucleation and assembly.

P153/B157

CLASP Modulates the GTP-Cap Length of Plant Cortical Microtubules.

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Flowering plants form distinct cortical microtubule array patterns in the absence of centrosomes that guide the deposition of cellulose into the cell wall. The single CLASP gene in *Arabidopsis* contains 3 tog domains, similar to human CLASP2, but lacks the SxIP EB1 binding sites. The *clasp1* mutant exhibits cell morphology defects, slow reorganization of the interphase cortical microtubule patterns, and 30% fewer growing microtubule plus ends. To determine how CLASP acts on these cortical microtubules, we imaged EB1-GFP and GFP-Tubulin in wild type and *clasp1* cells at 10 fps and applied new tracking algorithms to identify polymer growth properties. Wild type and *clasp1* microtubules both exhibit episodic pausing in *vivo*. The switch from microtubule growth to shortening (catastrophe) was preceded in wild type and mutant by pausing and exactly correlated with the processive loss of EB1-GFP marking the GTP-cap. Super-resolution imaging indicates a 30% shorter EB1-GFP distribution on *clasp1* microtubule ends compared to wild type, correlated with the faster pause-to-catastrophe times for *clasp1* microtubules. Using mean subunit assembly rates and pause-to-catastrophe times, we estimate rate constants for GTP cap subunit transitions with CLASP and without CLASP. Fluorescence recovery after photobleaching of EB1-GFP indicates a slower recovery in the absence of CLASP. Examination of microtubule nucleation and rescue events indicates that CLASP acts generally to extend the GTP cap, leading to fewer catastrophe events and a higher probability of success for nascent growing plus ends during nucleation and rescue. Our results suggest that CLASP is acting generally to increase the lifetime of microtubules without substantially altering growth rates in this natively treadmilling system.

P154/B158

Septin 2/6/7 Complexes Tune Microtubule Plus End Growth and EB1 Binding in a Concentration- and Filament-dependent Manner.

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Regulation of microtubule dynamics is critical for many biological functions ranging from cellular morphogenesis and intracellular transport to cell migration and accurate chromosome segregation during cell division. Septins are GTP-binding proteins that organize into higher order complexes. Septins associate with the actin and microtubule cytoskeleton and have been shown to affect MT organization-

dynamics and post-translational modifications (e.g. Acetylation, tyrosination) in various cell types, but how septins impact the dynamic instability of MTs is poorly understood. Here, we reconstituted in vitro MT dynamics using total internal reflection fluorescence (TIRF) microscopy in the presence of recombinant SEPT2/6/7 complexes which comprise the minimal subunits of septin heteromers. We found that SEPT2/6/7 has a biphasic concentration-dependent effect on MT dynamics. Lower concentrations of SEPT2/6/7 enhance MT plus end growth and elongation, while higher and intermediate concentrations inhibit and pause plus end growth, respectively. We show that SEPT2/6/7 associates with higher preference to GTP- over GDP-bound MT lattice, and competes with End-Binding protein 1 (EB1) for binding to GTPγS-stabilized MTs, which mimic the EB1-preferred GDP-Pi state of polymerized tubulin. Notably, SEPT2/6/7 inhibits MT plus end binding and tracking of EB1 in cis by binding to the MT lattice and in trans when MT plus ends come in contact with SEPT2/6/7 filaments. SEPT2/6/7 filaments are more potent than actin filaments in pausing MT plus ends and dissociating EB1 from MT plus end tips in vitro as well as in cultured rat embryonic hippocampal neurons. Collectively, our data demonstrate that SEPT2/6/7 complexes and filaments can directly regulate MT plus end dynamics and the binding of plus end-binding proteins, and thereby promote the capture and invasion of MTs at intracellular sites or structures of septin enrichment.

P155/B159

Multi-scale Microrheology Using Fluctuating Semiflexible Filaments as Stealth Probes.

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The cytoskeleton governs cellular dynamics and provides structural support. These functions rely on the complex mechanical properties of the cytoskeleton, which strongly depend on the length scale. To characterize such scale-dependent mechanical properties in viscoelastic media, we propose a new method of microrheology using semi-flexible filaments. Such probes are much less invasive than the often-used micrometer-sized beads. By tracking multiple transverse bending modes we can simultaneously determine the micromechanical response of the medium on multiple length scales. We use single-walled carbon nanotubes (SWNT) as probes that can be accurately imaged based on their non-photobleaching fluorescence. We find that the viscoelastic properties of proof-of-concept test samples, sugar solutions and polymer solutions, measured in this way are in good agreement with those measured by conventional micro/macrorheology.

P156/B160

Direct Measurement of Force Production by Microtubules Disassembling in the Presence of Stabilizing and Destabilizing Agents.

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Microtubule-based pulling forces are the basis for chromosome movements during mitosis. A popular view is that strain energy released during microtubule disassembly, when straightened protofilaments in the lattice curl outwardly to form “ram’s horns” at disassembling plus-ends, imparts a significant amount of mechanical force crucial for chromosome movement. Additionally, this conformational wave of curling protofilaments is implicated in helping kinetochores to remain associated with disassembling microtubule tips. However, the fundamental mechanisms that govern the extent of protofilament

curling at disassembling tips remain unclear, and whether these curls affect kinetochore function has never been proven. Using a feedback-controlled laser trap, we are measuring the nanometer-scale displacements of microbeads as they are pushed laterally by curling protofilaments during disassembly. Previous work suggests that the extent of movement in this 'wave' assay might depend on how the bead is tethered to the microtubule wall. Therefore we are testing several different tethering methods, including a direct biotin-streptavidin linker, an anti-His antibody bound to 6xHis on the C-terminus of β-tubulin, and a DNA-based linker. To explore the relationship between wave energy, disassembly speed, and tip conformation, we are adding agents known to either speed disassembly and enhance protofilament curling (e.g., destabilizers such as Mg^{2+}, and MCAK) or slow disassembly and decrease curling (e.g., stabilizers such as taxol, or DZ-2384). This work will reveal how changes in microtubule disassembly conditions alter conformational wave energy, and will provide a first step toward understanding how wave energy influences kinetochore function.

P157/B161

Parameter Space Compression of Microtubule Dynamic Instability.

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Physical models of biological systems can become difficult to interpret when they have a large number of parameters. But the models themselves actually depend on (i.e. Are sensitive to) only a subset of those parameters. Rigorously identifying this subset of "stiff" parameters has been made possible by the development of parameter space compression (PSC). However, PSC has only been applied to analytically-solvable physical models. We have generalized this powerful method by developing a numerical approach to PSC that can be applied to any computational model. We validated our method against analytically-solvable models of random walk with drift and protein production and degradation. We then applied our method to an active area of biophysics research, namely to a simple computational model of microtubule dynamic instability. Such models have become increasingly complex, perhaps unnecessarily. By adding two new parameters that account for prominent structural features of microtubules, we identify one that can be "compressed away" (the "seam" in the microtubule) and another that is essential to model performance (the "tapering" of microtubule ends). Furthermore, we show that the microtubule model has an underlying, low-dimensional structure that explains the vast majority of our experimental data. We argue that numerical PSC can identify the low-dimensional structure of any computational model in biophysics. The low-dimensional structure of a model is easier to interpret and identifies the mechanisms and experiments that best characterize the system.

P158/B162

Microtubules Are Necessary for Proper Reticulon Localization during Mitosis.

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During mitosis, the structure of the Endoplasmic Reticulum (ER) displays dramatic reorganization and remodeling, however the mechanism driving these changes is poorly understood. Recently, the Reticulon family of ER shaping proteins has been identified as possible factors to promote these drastic changes in ER morphology. Here, we examined the role of cytoskeletal factors involved in the dynamics of a *Drosophila* Reticulon, Reticulon-like 1 (Rtnl1) during mitosis in the early embryo. At prometaphase, Rtnl1 localizes to the spindle poles just prior to the bulk of ER localization suggesting a role in

recruitment. Using precise temporal injections of cytoskeletal inhibitors in the early syncytial *Drosophila* embryo, we show that microtubules, not microfilaments are necessary for proper Rtnl1 localization during mitosis. Lastly, we show that astral microtubules are necessary for Rtnl1 localization to spindle poles early in mitosis and independently of the minus-end directed motor protein Dynein. This work highlights the role of the microtubule cytoskeleton in Rtnl1 localization to spindles during mitosis and sheds light on a pathway towards inheritance of the ER.

P159/B163

Investigating the Dynamics of Chromatin-driven Mitotic Spindle Assembly in *Drosophila Melanogaster*.

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Mitosis is the process by which duplicated chromosomes are separated between two daughter nuclei. This is facilitated by the mitotic spindle, a bipolar scaffold of microtubules (MTs) which organises and segregates the chromosomes. The formation of a robust mitotic spindle is important for the accurate segregation of chromosomes, and as such, MTs are generated in several different ways. Generally, the mitotic spindle is thought to be generated predominantly from centrosomal MT nucleation; however, abrogation of centrosomal MT nucleation results in a mitotic spindle generated predominantly from chromatin. Chromatin-driven mitotic spindle formation is known to rely on a RanGTP gradient generated in the vicinity of chromatin, and a number of spindle assembly factors (SAFs), including the Augmin (HAUS) complex and Mars (Hepatoma upregulated protein). This project aims to further elucidate the roles of SAFs in chromatin-driven mitotic spindle assembly using the *Drosophila melanogaster* syncytial blastoderm.

P160/B164

Microtubule Growth Rates Are Sensitive to Changes in Microtubule Plus-end Density.

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Microtubules (MTs) are inherently dynamic filamentous polymers that can stochastically switch between periods of growth (via tubulin subunit addition) and periods of shortening (via tubulin subunit subtraction). The process of MT plus-end growth has been shown to be important in establishing the steady-state dimensions of large MT assemblies such as the mitotic spindle (Reber et al., 2013; Milunovic-Jevtic et al., 2018; Lacroix et al., 2018), yet the fundamental mechanisms that underlie the spatial and temporal regulation of individual MT growth rates within the cell are still being characterized. Previous investigations into the regulation of MT growth rates were focused primarily on the molecular identities of key microtubule associated proteins (MAPs) and the protein composition of the cytosol. As such, these studies largely neglected the role of microtubule spatial density and growing MT plus-end competition in regulating MT growth rates. Here, we use fluorescence microscopy and geometrically confined *Xenopus* cell-free extracts to elucidate a previously un-characterized role for MT

plus-end density in regulating MT growth rates. Our analyses revealed that global MT growth rates in confined cytoplasm decrease as MT plus-end density increases. Moreover, we found that spatial differences in MT plus-end densities can impart local changes to MT growth rates within a contiguous cytoplasm. We speculate that these density-dependent effects likely exist due to limitations in the diffusive rate of key growth promoting factors (i.e. XMAP215, +TIP proteins). As a result, a growing MT system can experience a global depletion of components, while an individual growing MT end experiences a local protein gradient dictated by the presence and proximity of other growing MT plus-ends. These data suggest that individual MT growing ends compete for a limited pool of structural components or growth promoting factors.

Assembly and Disassembly of Cilia/Flagella

P161/B166

Cilia Development in Zebrafish Organ of Asymmetry.

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In vitro studies have identified two methods for ciliogenesis. The first, the centrosome/basal body constructs a cilium in the cell body where it then fuses with the plasma membrane. The second, the centrosome/basal body docks at the plasma membrane where it then assembles a cilium. The goal of our studies is to identify during in *vivo* organogenesis how cells construct a cilium. We have chosen to examine Kupffer's Vesicle (KV) development of *Danio rerio* (zebrafish) due to the transparency of the zebrafish embryo for live-cell 4-dimensional imaging. KV is an organ of asymmetry that is required to place visceral and abdominal organs with respect to the two main body axes. KV development requires cells to self-assemble into a rosette where cells then establish apicobasal polarity termed apical clustering. The rosette then transitions into a sphere with a fluid filled lumen. At this stage, cilia are projected into the lumen. Our studies have identified that early on in KV development, before a lumen has formed, cilia are being constructed in the cell volume (100%). In later stages of KV development (lumen expansion), we only see 1.3% of cilia still in the cell volume where the remaining population is projected into the lumen. These studies suggest that cilia first form in the cell body and then protrude into the extracellular space once a lumen is formed. To further identify the molecular mechanisms required to generate KV cilia we are analyzing the role of apically localized actin and the small GTPases Rab11 and Rab8. We propose a model that after the cilia is constructed and surrounded by a ciliary membrane cap, actin is organized along the apical membrane where it can generate tension to regulate when a cilium should protrude into the lumen. Based on the literature and our preliminary studies, Rab11-endosomes likely contribute to the formation of a ciliary cap and the recruitment of Rab8 that is required for cilia elongation. In efforts to further understand the role of Rab11 and Rab8 in ciliogenesis, we are utilizing an optogenetic approach to disrupt either Rab11 or Rab8 and quantify its effect on cilia development and maturation in vivo.

P162/B167

Conventional Actin Is Required for Sensing Deciliation Or Initial Execution of the Ciliary Re-assembly Program Post-deciliation In*Chlamydomonas Reinhardtii*.

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Chlamydomonas reinhardtii, a biflagellate unicellular green alga, is a powerful model for understanding human disease as its cilia closely resemble those in human cells.*Chlamydomonas* has two actin genes coding for a conventional actin (IDA5) and a novel actin-like protein (NAP1). Our previous studies show that after deciliation through acidic pH shock, cilia can be fully reassembled when either actin is present. However, when both actins are disrupted (by depolymerizing IDA5 with latrunculin B, LatB, in the *nap1* mutant background), ciliary reassembly is inhibited, and cilia do not reach full length. This is due to multiple roles for actins including in the transport post-Golgi vesicles. Surprisingly, our new results show that total actin disruption during only the 10 minute period prior to and during deciliation also causes significant ciliary assembly defects. Further, impaired ciliary assembly is robust in both *nap1* mutants and wild-type cells with extended LatB pre-treatment (a condition in which NAP1 is typically upregulated and can rescue most IDA5 phenotypes). This indicates that NAP1 upregulation cannot compensate for actin functions before/during deciliation. Prior work in *Chlamydomonas* suggests deciliation is followed by a signal to the cell that it needs to upregulate the production of ciliary proteins to begin to rebuild cilia. When we acutely disrupt both actins only during the short period prior to and during deciliation, cilia behave similarly to cells treated with the protein synthesis inhibitor cycloheximide, which prevents cells from incorporating any new proteins that would have been synthesized as a result of deciliation. Our findings suggest that the presence of actin is somehow necessary for the cilia to either propagate the deciliation signal, or to initiate the subsequent process of ciliary protein upregulation.

P163/B168

Speed and Diffusion of Kinesin-2 Are Competing Limiting Factors in Flagellar Length Control Model.

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Flagellar length control in Chlamydomonas is a tractable model system for studying the general question of organelle size regulation. We have previously proposed that diffusive return of the kinesin motor that powers intraflagellar transport can play a key role in length regulation. Here we explore how the motor speed and diffusion coefficient for the return of kinesin-2 affect flagellar growth kinetics. We find that the system can exist in two distinct regimes, one dominated by motor speed and one by diffusion coefficient. Depending on length, a flagellum can switch between these regimes. Our results indicate that mutations can affect length in distinct ways. We discuss our theory's implication for flagellar growth influenced by beating and provide possible explanations for the experimental observation that a beating flagellum is usually longer than its immotile mutant. These results demonstrate how our simple model can suggest explanations for mutant phenotypes.

P164/B169

Analysis of Protein Dynamics in Motile Cilia of Mouse Tracheal Epithelial Cells Using High-resolution Live Imaging.

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Dysfunction of motile cilia results in defects in fluid clearance and the pediatric syndrome primary ciliary dyskinesia (PCD). Motile cilia possess a 9+2 microtubule structure with nine outer microtubule doublets surrounding a central pair apparatus. While the importance of the motile cilia in human health is clear, the ability to study and understand protein dynamics in mammalian motile cilia has been limited, largely based on availability of useful tools and feasible strategies. In this study, we demonstrate a novel application of high-resolution, live confocal imaging to analyze ciliary protein trafficking and dynamics in ciliated mouse tracheal epithelial cells (mTECs). The mTECs are cultured at an air-liquid interface to enable optimal ciliogenesis, and GFP-tagged ciliary proteins are over-expressed using a lentiviral system. We have performed live single molecule imaging of GFP-tagged intraflagellar transport protein 52 (IFT52) in mTECs from wild type mice and a PCD mouse model with a mutation in the central pair apparatus gene *CFAP54*. Quantitative analysis by single molecule tracking and image correlation spectroscopy enables an in-depth interrogation of protein dynamics within the intraflagellar transport system of mammalian motile cilia. This study establishes a powerful platform for analysis of protein dynamics to elucidate the cellular mechanisms underlying mammalian motile cilia and PCD pathogenesis.

P165/B170

Microtubule Inner Proteins Weave into the Doublet Microtubule Tubulin Lattice.

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As the hair-like protrusions that beat at high frequencies to propel a cell or move fluid around the cell, cilia are both structurally flexible and stable. Little is known about how cilia are able to maintain its stability during high-frequency bending cycles. Cilia are composed of radially bundled doublet microtubules. The doublet microtubule is composed of a 13-protofilament A-tubule, a partial 10-protofilament B-tubule and microtubule inner proteins (MIPs) inside the tubulin lattice. In contrast to the cytoplasmic microtubule, the doublet microtubule does not show any dynamic instability. This prompts the question of how the MIPs influence the doublet microtubule stability and rigidity. The objective of the study is to uncover the molecular architecture of the tubulin lattice of the doublet microtubule and the effect of the MIPs on the tubulin lattice. Using single particle cryo-electron microscopy, we obtained the structure of the Tetrahymena doublet microtubules at 4.3 an gstrom resolution. Remarkably, the tubulin lattice of the doublet microtubule has a complex pattern in dimer distance and curvatures within the 48-nm repeat. This is due to an intricate network of MIPs, weaving into the tubulin lattice. In particular, the filamentous MIPs at the protofilament ribbon area insert itself into the interdimer interface every two tubulin dimers, leading to a distinct bimodal pattern of short and long dimer distances every 16-nm. The filamentous MIPs also form a head-to-tail interaction consecutively to form long chains along the protofilament ribbon and consequently, increase the stability and elasticity of the doublet. These results suggest that there is a complex interplay between

the MIPs and the tubulin lattice in the doublet microtubule. In addition, the doublet microtubule is mechanically stressed. This stress could be tweaked by the MIPs in the regulation of ciliary functions.

P166/B171

Outer Arm Dyneins Are Locked in a Closed State by Novel Factors Prior to Delivery to the Ciliary Axoneme.

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Cilia motility is vital in organisms ranging from protozoa to humans. Key drivers of this beating movement are outer arm dyneins (ODAs). Here we identify two novel factors that bind newly synthesised ODAs prior to their delivery to the axoneme. We purified ODAs from cell bodies of de-ciliated *Tetrahymena thermophila*. Negative stain electron microscopy showed these ODAs are locked in a closed conformation in contrast to the open bouquet of ODAs purified from axonemes. Mass spectrometry identified two proteins (Q22YU3 and Q22MS1) that co-purify with cell body ODAs. We show that incubation with recombinant Q22YU3 and Q22MS1 is sufficient to induce closure of the ODA bouquet. We propose that closed ODAs represent an inhibited pre-assembled state. The newly discovered proteins provide a molecular explanation for how ODAs are prevented from moving on microtubules in the cell body prior to their delivery to the ciliary axoneme.

P167/B172

Testing the Ion-current Model of Flagellar Length-sensing.

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The mechanism by which cells control size of organelles is a fundamental question in cell biology about which very little is known, in part because the complexity of many organelles makes their size control mechanisms difficult to study. Eukaryotic cilia and flagella are microtubule-based organelles whose relatively simple structure makes them ideal for investigating the mechanisms of size regulation. Flagella are constructed and maintained via an active transport process called intraflagellar transport (IFT). Since no protein synthesis occurs in the flagellum, new materials must be injected into the flagellum by a process called IFT injection. The rate of IFT injection has been shown to negatively correlate with flagellar length. This length-dependent IFT injection rate suggests that the cell has some method for measuring its flagella. However, it remains unknown how the cell measures the length of its flagella and controls IFT injection. Here we experimentally tested one theoretical model of flagellar length-sensing: the ion-current model. This model posits that there is a uniform distribution of calcium channels along the flagellum, and that the calcium current from the flagellum into the cell body increases linearly with flagellar length. In this model, the cell uses the calcium current to negatively regulate IFT injection. We used quantitative total-internal-reflection-fluorescence (TIRF) microscopy to measure the calcium level inside of *Chlamydomonas* flagella using a genetically encoded calcium indicator. The level of calcium inside of flagella was weakly correlated with the length of flagella. We then quantified IFT injection in wild-type and calcium-deficient flagella using TIRF microscopy. We found that the amount of IFT injection was reduced and decoupled from the length in calcium-deficient flagella. Thus, while calcium may be important for regulating IFT, it is not a simple negative regulator of IFT injection and could not

form the basis of a stable length control system. This observation does not support the ion-current model.

P168/B173

High-resolution Cryo-em Structure of the Decorated Ciliary Doublet Microtubule.

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Cilia are hair-like organelles present on the surface of almost every eukaryotic cell and are responsible for cellular motility, fluid flow and sensory perception including sight, hearing and smell. At the heart of each cilium is a highly conserved structure called the axoneme that spans the entire length of the organelle. It is the largest protein complex in many organisms and can be many times larger than a cell body. Unlike bacterial flagella, which consist of thousands of copies of a single protein, the axoneme is made from hundreds of different proteins. Scaffolding the axoneme are 9 doublet microtubules that are essential for the architectural rigidity and motility of cilia and provide a track for intraflagellar transport. Bound periodically along the length of each doublet microtubule are a variety of microtubule-associated proteins and complexes that decorate the external and luminal faces of the microtubules. These proteins remain in register along the entire length of the doublet microtubule. Furthermore, all doublets within an axoneme are in coherent register with one another. An y breakdown of this coherence leads to impaired motility. How periodicity is established, maintained, and synchronized for such large structures has been a long-standing mystery. In this study we determined the high-resolution structure of the 96-nm repeat unit of a native, fully decorated doublet microtubule and build a near-complete atomic model for its internal 48-nm repeat. The structure is one of the most complex ever solved by cryo-EM with 40 different proteins arranged into ~450 chains. Our structure demonstrates how these proteins establish the unique architecture of doublet microtubules, maintain coherent periodicities along the axoneme, and stabilize the microtubules against the repeated mechanical stress induced by ciliary motility. The structure will serve as a reference for interpreting genetic, biochemical, and physiological data from different cell types and for understanding the etiology of human ciliopathies.

P169/B174

Localization of FAP93 and PP2A at the Proximal End of *Chlamydomonas* Ciliary Axonemes.

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In most motile cilia, bending is initiated from the proximal end of the axoneme. Additionally, the proximal axoneme is also relatively stable compared to the distal axoneme (Alford et al., 2016). However, we do not have a detailed understanding of the specialized proteins localized to the proximal axoneme. Using *Chlamydomonas*, we performed iTRAQ proteomics analysis to quantitatively compare protein enrichment in short (~2µm) versus full-length (~11µm) axonemes. We predicted that specialized proteins localized at the proximal, or distal, axoneme will be enriched in the short axonemes (see Satish Tammana et al., 2013). Short axonemes were isolated from regenerating cilia {Hunter et al. 2016} or from the *pf9-2; pf28* double mutant which grows axonemes ~2-4µm long (Porter et al., 1992). We detected over 200 proteins enriched ≥1.4-fold in short axonemes. Consistent with previous reports, the minor dynein heavy chains (DHC3, DHC4, and DHC11) known to localize to the proximal axoneme

(Piperno, 1991; Yagi et al., 2009) and the tip protein FAP256 (CEP104) (Satish Tammana et al., 2013) were enriched in short axonemes. Our current focus is on two proteins the iTRAQ analysis revealed are enriched in short axonemes, FAP93 and PP2A. FAP93, a protein of unknown function, is enriched in the short *pf9-2; pf28* double mutant axonemes. Consistently, immunoblots show enrichment of FAP93 in short axonemes and immunofluorescence staining of FAP93 shows localization at the proximal axoneme. We are currently examining dynein assembly and stability of axonemes in newly isolated *fap93* null mutants. Unexpectedly, PP2A C and B subunits are enriched in both regenerating and *pf9-2; pf28* short axonemes. Consistent with this hypothesis, the *Chlamydomonas* motility mutant *pf4*, defective in the gene that encodes the PP2A B-subunit, has decreased swimming velocity {Elam et al., 2011}. The swimming phenotype was confirmed in newly isolated *pf4* null alleles. Immunoblot analysis also showed enrichment of PP2A-C and B subunits in short axonemes and immunofluorescence staining of PP2A3-HA (IMP3-HA, Lin et al., 2013) showed enriched localization at the proximal end of the axoneme. We postulate that PP2A, localized to the proximal 2µm of the axoneme, is a candidate component for regulation of ciliary bending.

P170/B175

Rhythmic Pulsation of Flagellar Axonemal Protein Pools in the Algal Cell Body.

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Trafficking into cilia and flagella through the gated entrance near the basal body (BB) entails elaborate mechanisms, including the motor-driven intraflagellar transport (IFT). Trafficking-related anomalies account for many congenital disorders. To elucidate upstream events, this study illuminated two types of flagellar cytoskeletal proteins in live alga Chlamydomonas. IFT-delivered proteins were enriched at the exclusion zone underneath the BBs where IFT trains congregated. This area was dimmer than flagella, and the intensity was inversely related to flagella lengths. Interestingly, this area pulsated rhythmically. In contrast, the IFT-independent FAP20, which bound to microtubule doublet junction - was enriched in the nucleus. Nonetheless, the nuclear area also pulsated, pushing fluorescent FAP20 toward the flagellar entrance via straightforward or meandering paths but bypassing the exclusion zone. Inverse relationship of osmolarity with pulsation frequency and flagellar generation suggested the involvement of the adjacent contractile vacuoles. In addition, both proteins displayed an array of patterns in the cytosol, suggesting regulated vesicular associations. These observations shed light on multiple long-standing questions on ciliogenesis.

P171/B176

Ift Trains Fragment into 'Carts' of IFT-A, -B, and Dynein at the Ciliary Tip.

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Intraflagellar transport (IFT) trains carry building blocks from the cell body into cilia. These trains are assembled at the ciliary base, move by anterograde IFT to the flagellar tip and return by retrograde IFT to the cell body (Wingfield et al. 2017). In *C. reinhardtii*, the anterograde trains possess layers of IFT-A, IFT-B and IFT dynein particles with distinct periodicities whereas the retrograde trains are more amorphous (Jordan et al. 2018). The remodeling of trains at the ciliary tip involves fragmentation of the

anterograde trains (Chien et al. 2017). However, this study is based on IFT dynein and the IFT-B1 protein IFT27-GFP. Thus, the degree of train fragmentation, e.g., into "IFT carts" consisting of IFT-A and B or into IFT-A, B1 and B2 subcomplexes or into individual IFT proteins, remains unknown. Understanding of this reorganization could shed light on the mechanisms of cargo unloading and IFT dynein activation at the tip. Here, we analyzed IFT dynamics at the ciliary tip utilizing fluorescent protein-tagged IFT-A, IFT-B1, IFT-B2, and IFT motors subunits. All IFT proteins dwell at the ciliary tip for ~2s and material from one anterograde train is distributed onto several retrograde trains. We estimate that the IFT pool at the tip correspond to 3-5 anterograde trains based off of photo-bleaching experiments. Two-color imaging of IFT140-sfGFP and IFT54-mScarlet indicates that IFT-A and B remain together during the reorganization. IFT-A and B proteins continued to travel together in BBS4-deficient cilia revealing that the BBSome is not critical for A-B association in *C. reinhardtii.* While also dwelling for ~2s, BBS4-GFP mostly diffused at the ciliary tip rather than being stationary as the IFT proteins suggesting that it is released from IFT trains. FRAP analysis of retrograde IFT at the tip revealed that IFT54-mScarlet and the IFT dynein subunit D1bLIC-GFP mostly remained together during turnaround Our data support a model of IFT turnaround in which anterograde trains fragment into carts or short strings of carts while IFT-A, B and IFT dynein remain associated.

P172/B177

Biological Noise in the *Chlamydomonas* Length Control System.

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How cells control the size of their organelles is a long-standing biological question. Furthermore, to what extent are organelles subject to noise? to answer these questions, we investigate noise in *Chlamydomonas* flagellar length control. Flagella represent the ideal organelle to study this question, as they have they are essentially one dimensional, so changes in length are unambiguous and can be represented by a single number. We find that *Chlamydomonas* flagellar lengths vary significantly due to intrinsic fluctuations within the flagella and extrinsic noise that varies between cells. Compared to cells with equal length flagella, cells with different length flagella are worse at swimming, but better at gliding, suggesting that there may be evolutionary pressure to tune intrinsic noise in length. Our results show that noise exists on the level of organelles which can affect cellular function.

P173/B178

Mechanistic Insight into Ciliary Signaling Regulation through Map Kinases.

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Ciliary length and regulation are dictated by intraflagellar transport (IFT) which transports building materials into cilia to regulate assembly. Recent research has revealed multiple molecular targets in the Mitogen Activated Protein Kinase (MAPK) pathway that regulate ciliary length in many different types of cells from photoreceptors to *C. elegans* sensory neuron cilia. However, the mechanism that connects how MAPK regulates this function is unclear. Previously we have found that BCI, [(E)-2-benzylidine-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one], a vertebrate MAP Kinase Phosphatase 3 inhibitor, increases phosphorylated MAPK, shortens flagella, and prevents regeneration of full-length flagella in *Chlamydomonas reinhardtii*. Here we show that BCI also prevents the GFP-tagged heterotrimeric kinesin II subunit, KAP, from entering into the cilia. Interestingly, its recruitment to the basal bodies remains unaffected even during regeneration following pH shock. This suggests a defect in protein entry that

may be the cause for shortened flagella. After also observing changes in localization of the transition zone marker, NPHP4, we show that normal NPHP4-HAC localization is lost with increasing concentrations of BCI. These results hint at a mechanism for MAPK signaling as it relates to ciliary assembly through restriction of ciliary protein gating.

P174/B179

Actin Dysregulation in *Chlamydomonas Reinhardtii* Lacking LF5p.

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Previous research by Lai-Wa Tam et al. has shown that Long Flagella 5 protein (LF5p) regulates cilia length in *Chlamydomonas reinhardtii* with mutants lacking this protein (*lf5-2)* having cilia that are about 1.5x times longer than normal. The kinase domain of LF5p has high protein homology to human cyclin-dependent kinase-like kinase 5 (CDKL5), which has been shown to interact with Rac1, a critical regulator of actin. LF5p has been shown to localize to the proximal region of cilia close to the region where filamentous actin is localized. *Chlamydomonas* contains two actin genes: conventional (IDA5) and a novel actin-like protein (NAP1). Based on the interaction that CDKL5 has with Rac1 and the localization of CDKL5-like LF5p, we hypothesize that LF5p may regulate *Chlamydomonas* actins. Further, given our previously identified roles for filamentous actin in ciliary assembly and maintenance, LF5p-associated actin dysregulation may account for the length phenotype observed in *lf5-2* mutants. While mid-cell actin is undisturbed in *lf5-2* mutants, we find actin-dependent phenotypes in the absence of LF5p. We find that *lf5-2* mutants are unable to successfully form fertilization tubules, which are IDA5 dependent filamentous actin-rich structures formed in gametes. The inability to form these structures suggests a potential IDA5 actin dysregulation when LF5p is absent from cells. In addition to this, we previously found that cells treated with actin depolymerizing Latrunculin B (Lat B) require the activity of NAP1 to assemble their cilia to full length following severing. During the NAP1-dependent assembly phase, Lat B treated *lf5-2* mutants regenerate their cilia much more slowly compared to the Lat B treated controls. We have also previously shown that disruption of actin filaments results in disrupted transition zones at the base of cilia and impaired protein incorporation in cilia. *lf5*-2 mutants have been shown (Lai-Wa Tam et al. 2012) to regenerate their cilia more slowly compared to WT cells under normal conditions. Our data showing actin dysregulation in *lf5-2* mutants may indicate that the slow regeneration of these mutants could be caused by an actin defect.

P175/B180

Microtubule Inner Protein (mip) Identification in *Tetrahymena Thermophila*.

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Modern advances in Cryo-ET show that some stable microtubules contain microtubule inner proteins (MIPs), which may aid in maintaining structural and functional integrity. Recently, RIB72A and RIB72B were identified as MIPs in the motile cilia of Tetrahymena thermophila. Loss of these proteins leads to ciliary defects and loss of multiple densities inside the axoneme microtubules. Mass spectrometry of the RIB72A/B double knockout strains led to identification of additional MIP candidate proteins. We are analyzing these candidate MIPs using cell biology and Cryo-ET to expand our knowledge of the identities, structures, and functions of the lumenal meshwork of proteins inside of microtubules.

P176/B181

Fap20 Localizes to Motile Cilia Doublet Microtubules and Is Necessary for Ciliary Function.

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Motile cilia are hair-like cellular appendages that undulate to move extracellular fluid. In humans, motile cilia move cerebrospinal fluid, clear mucus from the airway, and move eggs down the Fallopian tube. As such, defects in motile cilia beating cause severe human disorders like hydrocephalus, respiratory defects, and female infertility. The ability of motile cilia to beat relies heavily on its axoneme structure. Cilia axonemes are composed of stable doublet microtubules. How these unique doublet microtubules are built and stabilized is poorly understood. Cryo-electron tomography (Cryo-ET) has been used to investigate the structure of the doublet microtubules. From these studies densities on the inside of these doublet microtubules were identified, called microtubule inner proteins (MIPs). Recently Rib 72 has been identified as a MIP and is necessary for the localization of the majority of a-tubule MIPs. We used mass spectrometry to identify the missing a-tubule MIPs. From the mass spectrometry we identified Fap20 as a candidate MIP. In *Chlamydomonas,* Fap20 localizes to the inner junction of the a-tubule. We find that *Tetrahymena* Fap20, expressed under its native promoter, localizes to motile cilia. Genomic knock down of Fap20 results in reduced swim speed of the organism, which is indicative of ciliary beating defects. Our results highlight the interdependence of ciliary structure and function.

P177/B182

Fap67 Localizes to Motile Cilia Doublet Microtubules and Plays a Role in Ciliary Function.

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Motile cilia are microtubule-based, cellular appendages responsible for directed extracellular fluid flow. Motile cilia are present within the human brain, lungs, and female reproductive tract. Defects in motile cilia function have dire and wide-ranging implications for human health and disease, with defects ranging from epilepsy to infertility. Cilia are comprised of an axoneme consisting of modified doublet microtubules. The formation and structure of doublet microtubules is necessary for motile cilia function. Cryo-electron microscopy and tomography show that doublet microtubules are decorated with lumenal proteins called microtubule inner proteins (MIPs). These MIPs are essential for stabilizing the doublet microtubule structure and overall function of motile cilia, however, the role and identity of all MIPs have yet to be discovered. Mass spectrometry identifies *Tetrahymena* Fap67 as a potential ciliary MIP. Endogenously expressed Fap67 localizes to motile cilia. Overexpression of Fap67 fragments reveals that Fap67 localization is dependent on its N-terminal. Fap67 knock down strains show reduced swimming speeds, indicative of disrupted ciliary beating. Our results suggest that Fap67 is vital for proper ciliary function.

Centrosome Assembly and Functions 1

P178/B184

PCMD-1 Regulates Centrosome Assembly in *C.elegans*.

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Centrosomes are the major microtubule-organizing centers in animal cells that play an important role in spindle bipolarity, cell polarity and organelle positioning during cell division. These organelles lack any membrane and consist of an orthogonally arranged pair of centrioles surrounded by pericentriolar material (PCM). In *Caenorhabditis elegans*, the main scaffold protein is SPindle-Defective protein 5 (SPD-5, a homolog of the human CDK5RAP2), while its interacting partner SPindle-Defective protein 2 (SPD-2/ Cep192 in humans) and the Polo-Like-Kinase-1 (PLK-1, a homolog of the human PLK1) facilitate the formation of the scaffold. During interphase SPD-5 forms a thin layer around centrioles, whereas the SPD-5 containing scaffold expands in a PLK-1 dependent manner upon mitotic entry. This expansion is necessary for robust microtubule nucleation by the centrosome and the formation of a bipolar spindle. Here we present our current results on the previously unidentified centrosomal protein PeriCentriolar matrix deficient 1 (PCMD-1). We show that PCMD-1 localizes to centrosomes and is essential for cell division in *C. elegans.* We could show that the protein is required for the recruitment of pericentriolar core proteins SPD-5, SPD-2 and PLK-1 to the non-mitotic centrosome. In the absence of PCMD-1 the PCM cannot be stably formed and the integrity is disrupted. Thus, we speculate that PCMD-1 is an in *vivo* regulator of the PCM and acts as a platform for PCM proteins. We have shown that cellular levels of SPD-5 and SPD-2 remain unchanged in the absence of PCMD-1 and the proteins are not degraded on the cellular level, but not recruited to the centrosome. This finding supports the hypothesis that PCMD-1 might recruit these proteins through direct interactions. However, how exactly PCMD-1 interacts with other centrosomal proteins and how PCMD-1 itself is regulated is unknown. To identify interactions, we are currently performing different protein-protein interaction assays.

P179/B185

Plk1 Activity at Cenexin-positive Mitotic Centrosomes Is Required to Maintain Pericentriolar Material Architecture.

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The mitotic kinase, polo-like kinase 1 (PLK1), facilitates the assembly of the two mitotic spindle poles, which are required for the formation of the microtubule-based spindle that ensures appropriate chromosome distribution into the two forming daughter cells. Spindle poles are asymmetric in composition. One spindle pole contains the oldest mitotic centriole, the mother centriole, where the majority of cenexin, the mother centriole appendage protein and PLK1 binding partner, resides. We hypothesized that PLK1 activity is greater at the cenexin-positive older spindle pole. Our studies found that PLK1 asymmetrically localizes between spindle poles both in mammaliantissue culture and in Danio rerio (zebrafish) embryos under conditions of chromosome misalignment. Strikingly, chromosomes tend to misalign toward the oldest spindle pole in a cenexin- and PLK1-dependent manner. During chromosome misalignment, PLK1 activity is increased specifically at the oldest spindle pole, and this increase in activity is lost in cenexin-depleted cells. In the absence of cenexin, chromosome

misalignment increases towards both poles and correlates with fragmentation of the pericentriolar material (PCM) during metaphase. PCM fragmentation only occurs during metaphase and occurs asymmetrically, with more fragmentation occurring on the oldest spindle pole. These studies suggest that specific cenexin-mediated signaling events are required to maintain PCM architecture during metaphase. We propose a model where PLK1 activity elevates in response to misaligned chromosomes at the oldest spindle pole during metaphase where it can possibly adjust the function and organization of the PCM to assist in chromosome realignment.

P180/B186

Conserved Features and Functions of Centriolar Satellites.

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Centriolar satellites are cytoplasmic particles that cluster in the vicinity of centrosomes, the main microtubule organizing centers in animal cells. Despite extensive work linking satellites to various aspects of centrosome and cilium biology, their precise functional contribution continues to remain unclear. With mutations in satellite components linked to human developmental and adult disorders including microcephaly and ciliopathies, a better understanding of satellites is also of significant biomedical interest. Using reciprocal BLAST analysis we identified putative orthologs of the main satellite structural component PCM1 in both *Drosophila and C. elegans*, suggesting the distribution of PCM1 and potentially satellites may be wider than previously thought. The *Drosophila* homolog (CG10732, also known as Combover, Cmb) was originally described to be involved in planar cell polarity, with overexpression leading to disorganized wing hairs, a result we could confirm in our own hands. However, neither RNAi nor a putative loss of function mutant recapitulated this phenotype. Instead, loss of Cmb resulted in male sterility and uncoordination, potential ciliary defects. In order to further characterize Cmb we are currently examining Cmb protein localization, interactors and loss of function phenotypes at the light and electron microscopy level. In parallel, we are studying the function of the putative PCM1 ortholog in *C. elegans* by RNAi and CRISPR-mediated gene knock-out.By capitalizing on the power of the two invertebrate model organisms *Drosophila and C. elegans* we hope to be able to better define centriolar satellite function, dynamics and composition, essential to understanding their role in centrosome and cilium biology and consequently human health and disease.

P181/B187

Centriole Motility and Subcellular Positioning Require Pericentrin-like-protein and Kinesin-1.

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Centrosomes are the major microtubule organizing center (MTOC) of the cell. They comprise a pair of centrioles surrounded by a matrix of proteins term the pericentriolar material. Through microtubule nucleation they organize the mitotic spindle, cilia and flagella. To fulfill these functions, centrosomes must be motile to achieve proper positioning within the cell. Very little is understood about the different mechanisms of centrosome motility. Typically, it is thought to be governed by the activity of microtubule motors, pushing or pulling on the microtubules anchored at the centrosome. In some cell types, centrioles lack PCM and microtubules, and are otherwise known as inactive centrioles. Inactive centrioles must be motile as their intracellular positioning is critical for asymmetric cell division and sensory cilia formation. Despite this the mechanism by which inactive centrioles are able to move

around the cell is poorly described and not understood. Here we investigate how inactive centrioles move in interphase cells. Using high resolution live cell imaging coupled with genetic analysis in *Drosophila*, we show that centrioles move on microtubules, in a manner involved Dynein and Kinesin-1. Importantly, structured illumination microscope revealed Kinesin-1 localizes to centrioles in *Drosophila* S2 cells. To identify centriole components required for movement we performed a targeting RNAi screen and found that the centriole protein Pericentrin like protein (Plp) is necessary for centriole movement in interphase cells. Through yeast-2-hybrid analysis coupled with an in *vivo* interaction assay we mapped novel and direct protein-protein interactions between Plp and Kinesin-1. Structural analysis demonstrated that these interactions involve the conserved PACT domain. Our data support a model where Plp acts as a direct adaptor that links the centriole to motor proteins, thereby facilitating their movement. In this work we propose the first detailed mechanism of how centrioles can move independently of their role as an MTOC, in the context of developing tissue. Further understanding of inactive centriole motility has far-reaching implications in studies of asymmetric cell division and sensory ciliogenesis.

P182/B188

Analysis of the Salt-insoluble Scaffold within the Pericentriolar Material.

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Centrosomes are formed by the recruitment of microtubule-organizing pericentriolar material (PCM) to a pair of centrioles whose duplication once per cell cycle ensures the formation of a bipolar spindle during cell division. While the molecular pathways underlying centriole duplication are increasingly well understood, the nature of the pericentriolar material and how it is recruited to centrioles remains an area of active investigation.Classical experiments in a variety of experimental models revealed the existence of a filamentous matrix within the PCM that serves as a scaffold for the recruitment of other proteins that nucleate and anchor microtubules. The molecular composition of this scaffold, thought to be central to centrosome assembly, has not been determined. Functional experiments in *Drosophila and C. elegans* have put forward the coiled coil proteins Cnn and SPD-5, as candidates for scaffold components in their respective species. However, their presence in scaffold preparation has not been confirmed. Here, we isolate centrosomes from early-stage *Drosophila and C. elegans* embryos. As previously described, treatment with the chaotropic agents 2M KI and 6M GU strips away much of the PCM as well as centrioles, leaving behind a salt-insoluble scaffold incapable of nucleating microtubules. At least in *Drosophila*, incubation with cytoplasmic extracts results in the recovery of PCM components and restoration of microtubule nucleation potential. Using mass-spectrometry and quantitative immunofluorescence we have identified proteins enriched in scaffold preparations relative to intact centrosomes, amongst them Cnn and SPD-5, but also additional components in each system. The identification of matrix scaffolds in a wide variety of species implies a universal mechanism for centrosome assembly conserved across metazoans. We believe the molecular characterization of these scaffolds will be of particular interest given ongoing in *vitro* reconstitution experiments in both *Drosophila and C. elegans*.

P183/B189

The Formation of *Bona Fide* Centriole Wall Is a Prerogative for the Centriole-to-centrosome Conversion.

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The centrosome is the main microtubule-organizing centre (MTOC) of higher eukaryotic cells. It impacts on cell architecture, dynamics and cell-cycle progression, thus on tissue organisation and organism development. The first stage of centrosome inheritance is the duplication of its centriole core before the pericentriolar material (PCM) assembles around the new centriole in a maturation step termed centriole-to-centrosome conversion (CCC). It was proposed that CEP295-dependent recruitment of PCM proteins from the outside of the centriole is the first step of CCC. We show, based on the analysis of proteins that promote centriole biogenesis and centriole structure analysis via electron microscopy, that the developing centriole structure drives CCC. Depletion of the inner centriole protein CEP44 that interacts with POC1B and binds to the inner A-microtubules affects centriole structural organisation and CCC downstream of CEP295. Impairment of POC1B, TUBE1 or TUBD1, that compromised centriole integrity, also prevents CCC. Thus, formation of *bona fide* centriole via the CEP295, CEP44, POC1B, TUBE1 and TUBD1 centriole biogenesis cascade is a principle determinant for CCC.

P184/B190

Differential Requirements for Centrioles in Mitotic Centrosome Growth and Maintenance.

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Centrosomes, the predominant sites of microtubule nucleation and anchorage, coordinate spindle assembly and cell division in animal cells. At the onset of mitosis, centrioles accumulate microtubule-organizing pericentriolar material (PCM) in a process termed centrosome maturation. To what extent centrosome maturation depends on the continued activity of mitotic regulators or the presence of centrioles has hitherto been unclear. Using the *C. elegans* early embryo, we show that PCM expansion requires the Polo-like kinase PLK-1 and CEP192 (SPD-2 in *C. elegans*), but not its upstream regulator Aurora a (AIR-1), while maintenance of the PCM polymer depends exclusively on PLK-1. SPD-2 and PLK-1 are highly concentrated at centrioles. Unexpectedly, laser microsurgery reveals that while centrioles are required for PCM recruitment and centrosome structural integrity they are dispensable for PCM maintenance. We propose a model whereby centrioles promote centrosome maturation by recruiting PLK-1, but subsequent maintenance occurs via PLK-1 acting directly within the PCM.

P185/B191

Pericentrin Reduction from Basal Bodies Is Required for Sperm Development.

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Centrosomes are an intriguing sub-cellular structure that nucleates and organizes microtubules (MT) and establishes basal bodies to build axonemes, cilia and flagella. Centrosomes are assembled from a pair of centrioles surrounded by pericentriolar material (PCM). Centrioles and PCM are linked through

bridge proteins that include Pericentrin, Asterless, Spd2 and Sas4, which promote PCM accumulation in co-ordination with Polo kinase during mitotic onset. As cells exit mitosis, centrosomes undergo a process known as 'Centrosome Dematuration' (CD) where the amount of PCM drops to much lower interphase levels. Our study here focuses on a much less-known process referred to as 'Centrosome Reduction' (CR), which is similar to CD but specifically occurs following meiosis and during sperm development. We first carefully documented the entire process of CR in post meiocytes and discovered that their centrioles (basal bodies) undergo a sequential removal of PCM and bridge proteins during spermatid development. We noticed that Pericentrin is reduced soon after PCM (Centrosomin and Gamma-Tubulin) is removed from the centrioles of early spermatids. Given this observation or sequential reduction and the known role of Pericentrin in male fertility, we focused our study on the mechanism of Pericentrin reduction. We began by performing structure-function analysis of Pericentrin in post-meiotic male germline cells and in dividing wing disc cells. Based on numerous Pericentrin protein truncations, we found that N terminal deletions prevented Pericentrin reduction from the centrioles, which in turn perturbed proper spermatid development and male fertility. Further biochemical characterization revealed that Pericentrin N terminus region consisting of a strong protein degradation signals. We performed genetic and proteomic screens to identify the mechanism of Pericentrin degradation, which revealed specific targets that are linked to N-end rule degradation pathway. Furthermore, we found that eliminating the N terminal degradation signals also perturbed CD in cycling wing disc cells. This ectopic interphase PCM assembly is capable of nucleating MTs, independent of Polo kinase activity. Collectively, our results therefore suggest that regulation of Pericentrin degradation is part of the underlying molecular mechanism that promotes Pericentrin reduction in developing basal bodies and restricts interphase PCM recruitment in dividing cells.

P186/B192

Phase Separation and Innate Self-assembling Capacity of Pericentriolar Scaffold Proteins Drive the Formation of a Cylindrical Architecture at Human Centrosome.

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The centrosome is a membraneless organelle composed of two microtubule-derived structures called centrioles and an amorphous mass of pericentriolar material (PCM). Super-resolution microscopic analyses revealed that diverse PCM proteins are concentrically localized around a centriole in a highly organized manner. We previously reported that two pericentriolar scaffold proteins, Cep63 and Cep152, interact with each other to self-assemble into a higher-order cylindrical architecture around a centriole. However, mechanisms underlying how Cep63 and Cep152 reach their threshold concentrations in the vast cytosolic space of a cell to induce the self-assembled architecture remains a mystery. Here we observed that ectopically expressed Cep63 and Cep152 cooperatively generate amorphous cytosolic aggregates that exhibit dynamic turnover of its photobleached components with those in the surroundings within a few minutes. Intriguingly, these Cep63-Cep152 aggregates efficiently underwent fusion in vivo, suggesting that they possess physicochemical properties found in liquid-like assemblies. Studies with purified recombinant proteins showed that the Cep63-Cep152 complex is capable of generating either a cylindrical structure or a hollow sphere depending on the presence or absence of spatial cues. Unexpectedly, the complex also efficiently formed condensate-like solid spheres upon providing 5% polyethylene glycol as a molecular crowder, suggesting that it has a versatile capacity in adopting morphologically distinct higher-order structures. While their internal rearrangement abilities appeared to be somewhat limited, these in vitro assemblies also underwent dynamic turnover, implying

that the phase-separating activity of Cep63-Cep152 condensates is likely critical for generating a higher-order architecture. In line with this view, pericentriolar localization of endogenous Cep63 and Cep152 was sensitive to 1,6-hexanediol, a weak hydrophobic interaction-disrupting agent proposed to distinguish between liquid-like and solid-like assemblies. Combined, these findings suggest that self-assembly of the Cep63-Cep152 complex is driven by the intrinsic property of the complex undergoing density transition and subsequent molecular interactions, and that these stepwise processes are likely important to properly self-assemble into a higher-order cylindrical architecture.

P187/B193

Macromolecular Crowding Stabilizes the *C. Elegans* Centrosome Scaffold.

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Centrosomes are membrane-less organelles that are important for mitotic cell division. By nucleating tubulin and providing anchoring sites, they act as biochemical hubs that resist microtubule pulling forces during chromosomal segregation. To achieve this, the centrosome must assemble an amorphous mass of protein called pericentriolar material (PCM). The extent to which macromolecular crowding plays a role in the morphology and stabilization of the PCM is poorly understood. Here, we used laser microsurgery to extrude centrosomes from C. elegans embryos and compared different buffer conditions for their ability to stabilize the PCM extracellularly. The PCM dissolves in all buffers but retains its full mass in conditions containing macromolecular crowders such as polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP). Our results suggest that macromolecular crowding is necessary for PCM stability and its intracellular regulation may be critical to achieve proper centrosomal form and function for cell division.

P188/B194

Autophagy Is Required for Centrosome Loss-mediated Cell Cycle Arrest.

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The centrosome is composed of two centrioles surrounded with the pericentriolar material (PCM). It serves as the microtubule-organizing center (MTOC) and as the template for cilia formation. It is known that centrosome loss leads to p53 activation and G1 arrest in non-transformed cells. However, the regulation that are involved in sensing and mediating centrosome loss-induced cell cycle arrest remians largely unclear. Here, we perform a SILAC-based proteomics analysis to examine the cell responses to centrosome loss and find that centrosome loss causes lysosomal accumuation in RPE1 cells. We also demonsdtrate that the accumation of lysosome in acentrosomal cells comes from the increase of autophagic flux, which is independ to p53 activity. ATG5 knockout cells are then generated to analzye the importance of autophagy in the control of cell responses to centrosome loss. Lack of autophagy promotes cell death when cells loss the centrosomes. Together, we uncover that autophagy is required for the centrosome loss-mediated cell cycle arrest in cycling cells.

P189/B195

***Drosophila Plp* Messenger RNA Localization and Translational Control.**

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The centrosome is an essential organelle that regulates cell division and ciliogenesis. Centrosomes comprise a proteinaceous matrix of pericentriolar material (PCM) that surrounds a pair of centrioles. *Drosophila* Pericentrin (Pcnt)-like protein (PLP) is a key component of the centrosome that functions as a scaffold for PCM assembly required for microtubule nucleation and centrosome function. The disruption of *plp* in *Drosophila* impairs PCM recruitment and organization, while the deregulation of Pcnt in humans is associated with MOPD II and Trisomy 21. We recently found *plp* mRNA localizes to the PCM of *Drosophila* centrosomes where it colocalizes with PLP protein. While RNA is known to associate with centrosomes in diverse cell types, whether the localized centrosomal mRNA undergoes on-site translation and the physiological significance of the mRNA localization to centrosomes remains completely unknown. mRNA localization and translational control is a conserved mechanism that regulates many cellular processes, including cell migration, cell-cycle progression, and the development of embryos and neurons. Control of mRNA localization and translation is mediated by RNA-binding proteins (RBPs). Importantly, we find that *plp* mRNA interacts with the RNA-binding protein Orb, and PLP protein expression is regulated by Orb. We aim to uncover whether local *plp* mRNA contributes to centrosome function during *Drosophila* embryo development.

P190/B196

***Centrocortin* Forms a Unique Centrosome-associated Granule in *Drosophila* Embryos That Regulates Cell Division.**

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Centrosomes are the primary microtubule nucleating center of animal cells and play critical roles in cellular organization. Well known as the builder of the mitotic spindle in dividing cells, centrosomes also direct cell migration, template cilia, and organize trafficking in interphase cells. Centrosomes are composed of a pair of microtubule-based centrioles, which are surrounded by a protein matrix termed the pericentriolar material (PCM). The PCM is responsible for nucleating microtubules, and changes to PCM structure and composition during the cell cycle regulate centrosome activity. While the protein components of the centrosome are well understood, data from multiple species have implicated localized RNA as an enigmatic centrosome component. We therefore hypothesized that centrosome-associated RNA dynamically regulates centrosomes and cell division. Using quantitative imaging and single molecule fluorescence in *situ* hybridization, we found that multiple mRNAs are significantly enriched at centrosomes in *Drosophila* early embryos. One centrosome-associated transcript, *centrocortin* (*cen*), forms a micron-scale granule adjacent to centrosomes in a process regulated by the cell cycle and during development. This granule is labeled by Cen protein, *cen* RNA, and the canonical stress granule marker Fmr1. We find that Fmr1 regulates the steady state levels of *cen* RNA and protein, leading to increased granule size in the absence of Fmr1, although the granule is not required for Cen translation. The centrosome scaffold protein Centrosomin is required for formation of this granule, indicating that centrosomes actively contribute to the formation of the *cen* granule. Mutations to *cen and Fmr1* cause similar cell division phenotypes, leading us to conclude that regulation of the *cen* granule is critical for error-free cell division. We will present our latest work investigating the regulation of this novel centrosome-associated RNA granule and its role in cell division.

P191/B197

Scaffold Assembly of Pericentriolar Material in *Caenorhabditis Elegans*.

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The centrosome is a major microtubule organizing center in animal cells. Centrosomes are composed of two centrioles and pericentriolar material (PCM). During mitosis, PCM dramatically expands and increases its microtubule nucleating activity in the process called centrosome maturation. In this process, the assembly and disassembly of PCM scaffold proteins are tightly linked to the cell cycle and spindle formation. *Caenorhabditis elegans* SPD-5 is a functional homolog of human CDK5RAP2 and *Drosophila* Centrosomin, which are the major PCM scaffold proteins controlled by phosphorylation by Polo-like kinases 1. To investigate the mechanism by which SPD-5 forms PCM scaffolds, we performed domain analysis of SPD-5 in *vivo*. We constructed worm strains expressing GFP-tagged various truncated versions of SPD-5 proteins by the CRSPR-Cas9 system. Live-imaging microscopy indicated that the C-terminal region of SPD-5 is sufficient to be recruited to PCM in the presence of the endogenous SPD-5, and to localizes at the centrioles in the absence of endogenous SPD-5. Yeast two hybrid assays indicate that the C-terminal region of SPD-5 interacts with the middle region of SPD-5. Interestingly, the C-terminal region of SPD-5 also interacts with a centriolar protein PCMD-1. These data indicate that SPD-5 proteins are recruited to centrioles through the interaction between the C-terminal region of SPD-5 and PCMD-1, and then expanded to the PCM scaffold by interacting with PLK-1 and other centrosome proteins. We also identified several centrosomal proteins that interact with PCMD-1. We will discuss the mechanism of PCM scaffold formation by SPD-5, and how it contributes to the centriole-centrosome conversion.

P192/B198

PCM1 Loss Disrupts Centriolar Satellites and Causes Genome Instability.

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Centriolar satellites are granular, fibrous structures that surround the centrosome and regulate movement of proteins to and from the centrosome. Their role in human cancer has not been reported. We analyzed genomic and transcriptomic alterations in the 44 genes known to localize to the centriolar satellites. Notably, genomic deletion of pericentriolar material 1 (PCM1) is the most common centriolar satellite gene alteration and occurs in up to 15% of human cancers depending on the disease site, with the most common sites being bladder, colorectal, endometrial, and prostate. PCM1 is the major defining protein of centriolar satellites, so the impact of PCM1 deletions in cancer was further pursued. We used CRISPR genome editing to knockout PCM1 in HeLa and RPE1 cells. In these knockout cells, centriolar satellites are disrupted, as evidence by dispersion of other centriolar satellite proteins such as CEP131 and CEP72. Interestingly, we find that PCM1 knockout increases the incidence of multipolar spindles in metaphase and lagging chromosomes in anaphase. We probed multiple potential mechanisms known to cause multipolarity, finding that loss of PCM1 results in disruption of centrosome integrity at the onset of metaphase. We conclude that PCM1 is important for centrosome cohesion when force is exerted on the centrosomes during mitosis. To assess whether this role of PCM1 in genomic stability is shared by other centriolar satellite proteins, we engineered CEP131 knockout cells. Concordantly, CEP131 knockout cells exhibit more multipolar spindles in metaphase. Taken together, these findings

demonstrate that PCM1-deleted cancers are more chromosomally unstable suggesting that PCM1 deletion drives carcinogenesis by increasing the incidence of multipolar spindles. Further, these results point to a broader role for centriolar satellites in maintaining genomic stability.

P193/B199

Sperm Head-tail Linkage Requires Restriction of Pericentriolar Material to the Proximal Centriole End.

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An essential requirement for flagellated sperm is the tight attachment of the head, containing the genetic material, to the tail, needed for swimming. A failure in this attachment leads to decapitated and decaudated spermatozoa (DDS), which results in sub-fertility. The centriole is key to this attachment, building the cilium from its distal end and embedding its proximal end in the nucleus. Surprisingly, the role of the centriole itself in establishing a connection to the nucleus had not been studied. Using the *Drosophila* spermatid as a model system, we discovered a centriole centered mechanism to ensure the proper attachment of the proximal end of the centriole to the nucleus. Soon after meiosis concludes, the proximal end of the centriole is moved into close apposition with the nucleus. Based on our live cell imaging and other published data, we propose a model where the microtubule (MT) motor dynein, localized on the surface of the nucleus, pulls on MTs emanating from the centriole to bring the centriole and nucleus together. During this process the pericentriolar material (PCM), which nucleates and anchors MTs, is enriched at the proximal end of the centriole. This PCM organizes MT from the proximal end to ensure that this end is captured by MT motors on the nucleus. Furthermore we found that the major regulator of PCM, PLP, is also localized to the proximal end. We show that PLP is essential for the localization of PCM and in turn the ability of the centriole to dock to the nucleus. Using single cell RNAseq to dissect in *silico* developing spermatocytes, we found that PLP is proximally restricted by limiting the availability of its mRNA to the earliest stages of centriole growth. Altering the timing of the availability of PLP protein to later stages of centriole construction resulted in mislocalization of PLP along the entire length of the centriole. Distal PLP was sufficient to recruit PCM and organize MTs along the entire length of the centriole. Thus, the position of PLP along the centriole is sufficient to instruct the position of PCM and MTs. Finally, using light and electron microscopy we show that when PCM is mislocalized to distal regions of the centriole, the side of the centriole, instead of the proximal end, is brought into contact with the nucleus. In many of these cases, establishment of a permanent centriole/nuclear attachment fails, resulting in a separation of the sperm head and tail or DDS.

P194/B200

Domain Characterization of Pericentrin Uncovers the Role of Its Intrinsically Disordered Regions in Mediating Phase Separation and Microtubule Nucleation in Human Cells.

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The centrosome is a membraneless organelle that serves as the main microtubule-organizing center (MTOC) in metazoan cells. It orchestrates critical cellular processes such as cell signaling and cell division. The centrosome consists of a pair of centrioles surrounded by a proteinaceous network of pericentriolar material (PCM). It is the PCM that nucleates microtubules (MTs) and thus dictates the MTOC activity of the centrosome. The PCM is a dynamic ensemble of proteins, many of which contain

extensive intrinsically disordered regions (IDRs) that do not fold into stable secondary or tertiary structures. How the PCM encompasses the centrioles to form a membraneless, micron-sized centrosome is not well understood, in part because the roles of the IDRs in many of the PCM proteins are poorly defined. IDRs are widespread in eukaryotic proteomes and are associated with a diverse array of functions, including the formation of membraneless organelles through phase separation. Here we use an inducible gene expression system in mammalian cells to dissect the roles of IDRs in pericentrin (PCNT), a conserved PCM component critical for PCM assembly and mitotic spindle organization in vertebrates. In silico predictions suggested that PCNT and its orthologous proteins have extensive IDRs at the N-terminus and a structured centrosome targeting PACT domain at the C-terminus. Through live cell imaging analyses, we found that multiple N-terminal, IDR-containing domains of human PCNT condense into liquid-like assembly in a concentration-dependent manner in cultured cells. In particular, a region containing the putative dynein-interacting domain forms spherical “condensates” that grow in size, coalesce, and move toward the centrosome in a MT-dependent manner. Fluorescence recovery after photobleaching analyses showed that the phase of PCNT condensates transitions from liquid- to gel-like states over time. We further found that PCNT condensates selectively recruit endogenous PCM components and are capable of nucleating MTs in cultured cells. We propose that anchoring PCNT molecules around the centriole through the C-terminal PACT domains drives their N-terminal IDRs to undergo crowding-induced phase separation at the centrosome; the resulting condensates selectively recruit additional PCM components to promote the assembly of PCM and the maintenance of centrosome integrity. We further speculate that the IDR, a prevalent sequence feature found in centrosomal proteins, plays critical roles in centrosome assembly, likely in part through mediating phase transition of PCM components.

P195/B201

Ppp1r35 Binds and Protects Rttn within the Cell.

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The centrosome is the microtubule organizing center of the cell that guides the formation of the bipolar spindle, which functions to partition DNA to each daughter cell during mitosis. Each centrosome is comprised of two perpendicularly arranged microtubule-based structures called centrioles. Centriole copy number is tightly controlled to ensure that only a bipolar spindle forms in mitosis. Too many or too few centrioles can promote mitotic errors that drive the formation of aneuploid cells, which is a hallmark of cancer. At the start of the cell cycle each cell inherits a pair of centrioles that duplicate once prior to the next division. While centriole duplication occurs in S-phase, the newly formed centriole is duplication incompetent until it passes through mitosis. This cell cycle control of centriole duplication is known as the centriole-to-centrosome conversion (Wang *et al.* 2011 *JCB)*. We recently performed a CRISPR screen that identified PPP1R35 as a positive regulator of centriole biogenesis. A recent study has shown that PPP1R35 is required to the centriole-to-centrosome conversion (but the function of the protein has not been fully characterized) (Fong *et al.* 2018. *MBoC*). Here, I show that PPP1R35 stabilizes and protects the centriolar protein RTTN, from being proteolytically cleaved. The goal of this work is to determine the biological significance of this cleavage event and establish whether this cleavage is required for the centriole-to centrosome conversion.

P196/B202

Prolonged Mitosis Results in Structurally Aberrant and Over-elongated Centrioles.

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Centrioles are nine-fold symmetrical microtubule-based structures that organize centrosomes in interphase and mitosis, and assemble cilia and flagella. Centriole's length is constant for a certain cell type, although it may be different among different organisms and among different cell types of the same organism. Centrioles of human cycling cells are ~400 nm long. Centriole aberrations, which can be numerical and structural, are common in pathologies like tumors. Overly long centrioles can nucleate more microtubules, perturb mitosis and tissue architecture. How cells regulate centriole size and what leads to centriole over-elongation in tumors is not understood. An alysis of centriole structure is difficult because it requires demanding electron microscopy. Here we employ expansion microscopy to study the origins of centriole structural aberrations in large populations of human cells. We discover that centrioles do not have elongation monitoring mechanism, which renders them prone to over-elongation especially during prolonged mitosis induced by various factors, importantly including supernumerary centrioles. We further identify that mitotic centriole over-elongation is dependent on mitotic Polo-like kinase 1 (Plk1), which we uncover as a novel regulator of centriole elongation in human cycling cells. While the insufficient level of Plk1 leads to the formation of short centrioles lacking a full set of microtubule triplets, its overactivity results in over-elongated and structurally aberrant centrioles. Our data helps explain the origin of structurally aberrant centrioles and why centriole numerical and structural defects coexist in tumors.

P197/B203

Expansion Microscopy for the analysis of Centrioles and Cilia.

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Human centrioles are sub-resolutional microtubule (MT)-based cylindrical structures that build centrosomes and cilia. Due to their small size (~230 x 400 nm), the analysis of centriole's ultrastructural characteristics requires super-resolution imaging methods and electron microscopy, which are laborious and unsuitable for the collection of large data sets to permit a rigorous statistical analysis. Additionally, centrosomal proteins tend to form aggregates which can easily be mistaken for bona-fide centrioles. This poses a major problem in the centrosome field, as the conclusions obtained by conventional microscopy are often left uncorroborated by ultrastructural analysis. Here we present an adaptation of magnified analysis of the proteome expansion microscopy method, to be used for a robust analysis of centriole number, duplication status, length, structural abnormalities and ciliation by conventional optical microscopes. The method allows the analysis of centrioles' structural features from large populations of adherent and nonadherent cells and multiciliated cultures. We validate the method using EM and super-resolution microscopy and show that it can be used as an affordable and reliable alternative to electron microscopy in the analysis of centrioles and cilia in various cell cultures.

P198/B204

Combining Experimental and Modeling Approaches to Study the Effects of a Transient Centrosome Amplification Event on Steady State Levels of Supernumerary Centrosomes in the Cell Population.

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Extra centrosomes promote chromosomal instability and promote invasion in *vitro*. Moreover, centrosome amplification is common in cancer and can drive tumorigenesis in mouse models. Genome doubling, leading to tetraploid cells, is also closely linked with cancer and has been shown to promote tumorigenesis. Importantly, most cells that become tetraploid concurrently acquire extra centrosomes - for example, cytokinesis failure generates a tetraploid daughter cell with twice the normal number of centrosomes. It has been proposed that, because of the prevalence of both phenomena in cancer, tetraploidization is a major route of centrosome amplification during tumorigenesis in *vivo*. Yet, recent studies have reported that tetraploid cells grown in *vitro* generally do not exhibit increased centrosome numbers compared to diploids. We have recently identified a mechanism (Baudoin et al., 2019, BioRxiV) that leads to loss of extra centrosomes in newly formed tetraploid cells. Specifically, we found that populations of newly formed tetraploid cells rapidly lose their extra centrosomes by a process of natural selection in which tetraploid cells that inherit a single centrosome during a bipolar division with asymmetric centrosome clustering (3:1) are favored for long-term survival. We have constructed a mathematical model that describes this process in a population dynamics framework. A basic model, assuming a negative selective pressure for cells with extra centrosomes due to their likelihood to undergo multipolar division, captures centrosome loss, but under-estimates the final (steady-state) fraction of cells containing extra centrosomes within reasonable parameter ranges. Particularly, this result is constrained by the experimentally observed rates of cytokinesis failure in our model system. We found that the presence of a population of cells that cluster their extra centrosomes into bipolar spindles with high efficiency ("super-clustering," or 'SC', cells) relieves this constraint and allows our model to match experimental results. We will present experimental evidence supporting the presence of SC cells, and discuss the implications of our model for understanding to role of tetraploidy in promoting centrosome amplification in tumors.

P199/B205

***Drosophila* Cep104 Is a Novel Component of the Centriole Distal Tip Complex.**

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Centrioles, the core components of the centrosome, are barrel-shaped organelles composed of microtubule bundles arranged in nine-fold, radial symmetry. Although the general structure is evolutionarily conserved, centriole architecture is incredibly diverse across species and even within different cells of an individual organism. Currently, we have a limited understanding of how centrioles are built as well as the need for such variety in centriole structure. Therefore, we aim to understand the molecular mechanisms that control centriole assembly. Centriole biogenesis begins when a procentriole nucleates orthogonally off of the proximal end of an existing mother centriole. After recruitment of microtubules to the procentriole, processive centriole growth then occurs from the daughter's distal end. Understanding how cells control centriole length is important because abnormally long centrioles spawn multiple daughters simultaneously or fragment into small functional units, resulting in

centrosome amplification and, consequently, chromosomal instability. The distal tip of the centriole is occupied by a conserved set of length-regulating proteins (Cep97, CP110, and Klp10A in *Drosophila*), which we have termed the Distal Tip Complex (DTC). This ill-defined complex regulates the growth of centriolar microtubules and, thus, centriole length. At present, we lack a mechanistic understanding of this complex, the identity of all its components, and how it is regulated. By BLASTing candidate proteins against the *Drosophila* proteome, we have identified Cep104 as a novel member of the DTC. Cep104 encodes a multi-domain protein including an N-terminal Jellyroll domain, a coiled-coil region, a zinc-finger array, and a single TOG domain - a conserved tubulin binding and polymerization module. Notably, fly Cep104 lacks a C-terminal EB1-binding motif, which is present in the human homolog, and does not tip track on microtubule plus-ends. Through a series of function/structure experiments, we have identified regions of Cep104 necessary for its oligomerization and distal tip localization. Using yeast 2-hybrid and co-immunoprecipitation, we have mapped the binding domains within Cep104, CP110, Cep97, and Klp10A, revealing a more complete interaction network of the DTC. Our findings suggest that Cep104 is a key member of the DTC and, thus, participates in the control of centriole growth.

P200/B206

Role of Cep128 in Formation of the Centriolar Subdistal Appendage.

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The mother centriole in a cell has two appendages, the distal appendage (DA) and subdistal appendage (SDA), which have roles in generating cilia and organizing the cellular microtubular network, respectively. In the knockout (KO) cells of Odf2, the component of the DA and SDA, both appendages simultaneously disappear. Here, using super-resolution structured illumination microscopy (SR-SIM), we found that the signal for GFP-tagged Odf2 overlapped considerably with that of immunofluorescently labeled Cep128. We further found that Cep128 knockdown (KD) caused the dissociation of other SDA components from the centriole, including centriolin, Ndel1, ninein, and Cep170, while Odf2 was still associated with the centriole. In contrast, the DA components remained associated with the centriole in Cep128 KD cells. Consistent with this observation, we identified Cep128 as an Odf2-interacting protein by immunoprecipitation. Taken with the finding that Cep128 deletion decreased the stability of centriolar microtubules, our results indicate that Cep128 associates with Odf2 in the hierarchical assembly of SDA components to elicit the microtubule-organizing function.

3

Cytokinesis 1

P201/B207

Oocyte Meiotic Spindles & Polar Body Extrusion: a Role for CLS-2/CLASP in Contractile Ring Assembly.

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During oocyte meiotic cell division, discarded chromosomes are extruded into small polar bodies. However, the cues that mediate polar body extrusion are not well understood. To gain insight, we are investigating the relationship between oocyte meiotic spindle assembly and polar body extrusion. We have begun by exploring the role of CLS-2, a *C. elegans* CLASP family member, and are comparing its

spindle assembly and polar body extrusion defects to those in *mei-1/Katanin* and *klp-18*/kinesin 12 mutants. Previous studies of CLASP family proteins indicate that they function to promote microtubule stability and may be important for regulating interactions between the microtubule and actin cytoskeletons, but how they contribute to polar body extrusion remains unknown. We used CRISPR/Cas9 to generate putative *cls-2* null alleles, and live imaging with fluorescent protein fusions to assess CLS-2/CLASP requirements. As others have reported, *cls-2(-)* mutants have chromosome segregation defects during oocyte meiosis. In addition, we have found that microtubule levels are reduced, and the mutant oocytes fail to assemble bipolar spindles. Intriguingly, ~85% of *cls-2(-)* oocytes fail to extrude a polar body during meiosis I (n=91) and exhibit increased levels of global cortical furrowing compared to wild type. Moreover, based on live imaging of NMY-2/non-muscle myosin and ANI-1/anillin, the polar body contractile ring structure appears fragmented and discontinuous in *cls-2(-)* mutants. Similar to *cls-2(-)* oocytes, *mei-1 and klp-18* mutant oocytes fail to segregate chromosomes, and *mei-1/Katanin* mutants also frequently fail to extrude a polar body during meiosis I (83%, n=23), although global cortical furrowing appears normal. In contrast, oocytes depleted of *klp-18/kinesin 12* usually do extrude polar bodies (15% failure in meiosis I, n = 20) and also have more normal global cortical furrowing. Unlike the fragmented and disorganized contractile ring structures observed in *cls-2(-)* mutants, oocytes lacking *mei-1/Katanin* and *klp-18/kinesin 12* form more normal contractile rings, though later scission appears to fail frequently in *mei-1* depleted oocytes. We conclude that CLS-2/CLASP has a unique and important role in contractile ring assembly during oocyte meiosis. We are currently analyzing spindle associated furrow dynamics, cortical non-muscle myosin dynamics, and spindle components to further compare spindle assembly and polar body extrusion defects in these and other mutants. We anticipate that such a comparative analysis will provide insight into the cues that mediate polar body extrusion during oocyte meiotic cell division.

P202/B208

The Ran Pathway Uniquely Regulates Cytokinesis Depending on Cell Fate in *Caenorhabditis elegans* Embryos.

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Cytokinesis is a highly conserved process that divides a cell into two daughters. This process must be tightly regulated to avoid aneuploidy or cell fate changes. Cytokinesis requires the assembly and ingression of a RhoA-dependent actomyosin ring that constricts to pinch in the overlying cortex. This process is regulated by microtubule-dependent and -independent mechanisms, and our lab found that a chromatin-sensing mechanism also regulates cytokinesis. In interphase cells, proteins with nuclear localization signals (NLS) are bound by importin-α/-β for import into the nucleus where they are released by Ran-GTP. RCC1/RanGEF is associated with chromatin in the nucleus, while RanGAP is in the cytosol forming a gradient of Ran-GTP that is retained during mitosis. An inverse gradient of importins free to bind to cargo forms with higher levels near the cortex, and we found that human anillin, a scaffold for the contractile ring, is regulated by importin-binding for its function in cytokinesis. Importin-β binds directly to a conserved NLS in the C-terminus of anillin to facilitate its cortical recruitment. While different mechanisms function redundantly to regulate cytokinesis in symmetrically dividing cells in *vitro*, their requirement in *vivo* likely varies depending on cell geometry, fate or ploidy. We studied the role of the Ran pathway in cytokinesis of cells of two different fates in the early *C. elegans* embryo. The first division gives rise to a larger anterior AB cell fated to give rise to multiple tissues, and a smaller posterior P_1 cell fated to become germline. Imaging AB and P_1 cell division from anaphase onset with

high temporal resolution revealed that each cell has unique ingression kinetics, supported by differences in the cortical accumulation of ANI-1 in the two cells. Lowering Ran-GTP levels via partial RNAi of RCC1 (RanGEF) increased kinetics of the early phases of cytokinesis, which was suppressed by co-depletion of contractility regulators such as ECT-2 (RhoAGEF) or LET-502 (Rho Kinase). Interestingly, co-depletion of ANI-1 suppressed RCC1 phenotypes in AB, but not P_1 cells, suggesting that the two cells have different pathway requirements. This was supported by differences in cytokinesis phenotypes caused by depletion of importin-α (IMA-3), -β (IMB-1) or both. We are currently testing this hypothesis using CRISPR to generate mutations in ANI-1 that disrupt IMB-1 binding. Thus, our findings reveal differences in mechanisms regulating cytokinesis in cells with different fates, and emphasize the need to study cytokinesis in cells in *vivo*.

P203/B209

Probing the Role of ESCRT-III Proteins in Cytokinetic Abscission and the Abscission Checkpoint.

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Cytokinetic abscission, the final stage of cell division, involves tight coordination and regulation of microtubule and membrane remodeling events along with finely-tuned dynamics in protein localization. A checkpoint just prior to this physical separation of daughter cells termed the abscission (or NoCut) checkpoint is thought to ensure that residual mitotic errors, such as malformed nuclear pores, chromatin bridges, or under-replicated DNA lesions, have been corrected before proceeding to the irreversible step of abscission. Thus, the abscission checkpoint helps maintain DNA integrity and ensures that nascent daughter nuclei are fully formed and poised for proper function. While it is well-established that Aurora B kinase is a master regulator of abscission timing and the checkpoint itself, questions still remain about the spatiotemporal regulation of protein recruitment and activities required to orchestrate midbody stabilization and its consequent resolution. Recent studies have found that certain subunits of the ESCRT membrane remodeling machinery play key roles in both abscission and the abscission checkpoint. To understand better how ESCRT proteins contribute to this step of cell division, we explored three specific ESCRT-III binding partners: SPASTIN, KATANIN p60, and CALPAIN7. In each case, we have characterized features that specify their MIT domain interactions with particular ESCRT-III proteins and tested their roles in abscission and abscission regulation. These studies reveal that distinct ESCRT-III factors target the microtubule severing proteins SPASTIN and KATANIN p60 to the midbody for distinct functions in abscission. Moreover, we unexpectedly found that SPASTIN is also required for the abscission checkpoint. We further discovered a previously uncharacterized role for the cysteine protease CALPAIN7 in the abscission checkpoint. The catalytic activities of both SPASTIN and CALPAIN7 are essential to their role in the abscission checkpoint, highlighting the variety of biochemical activities that contribute to abscission regulation.

P204/B210

Mammalian Cell Growth Dynamics in Mitosis.

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The extent and dynamics of animal cell biomass accumulation during mitosis are unknown, primarily because growth has not been quantified with sufficient precision and temporal resolution. To study

growth in mitosis, we use suspended microchannel resonator, a non-invasive mass sensor that allows us to quantify single-cell cell mass accumulation rates with 0.05 % precision and a temporal resolution of 1 min, while simultaneously monitoring fluorescent and biophysical markers of mitotic stages. We also support the findings of mass accumulation measurements with single-cell protein synthesis assays. We find that in various animal cell types, growth rates in prophase are commensurate with or higher than interphase growth rates. Mass accumulation is only stopped as cells approach metaphase-to-anaphase transition and mass accumulation resumes in late cytokinesis. Mitotic arrests stop growth independently of arresting mechanism. For mouse lymphoblast cells, growth in prophase is promoted by CDK1 through increased phosphorylation of 4E-BP1 and cap-dependent protein synthesis. Inhibition of CDK1-driven mitotic translation reduces daughter cell growth. Overall, our high-resolution approach to cell growth monitoring counters the traditional dogma that growth during mitosis is negligible and provide insight into antimitotic cancer chemotherapies.

P205/B211

Cytokinetic Bridge Supports Tensions Required for Rosette Formation*In Vivo*.

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Multicellular rosettes are cellular intermediates observed during the formation of diverse organ systems. Rosettes are polarized, transient epithelial structures that can be prerequisite to the development of an adult organ. A representative example of this process is the formation of Kupffer's Vesicle (KV) during development of *Danio rerio*(zebrafish),where non-polarized cells self-organize into a polarized rosette and then a sphere encircling a fluid-filled lumen. Once fully formed, KV establishes zebrafish left-right asymmetry and is a conserved process amongst vertebrate systems. Where most rosettes identified thus far employ either apical constriction or planar polarized constriction for their formation, we have identified a unique example of rosette assembly that incorporates the last stage of cell division, cytokinesis. Our studies address the question that is fundamental to understanding KV formation: What physical properties and biomechanical steps are required for cells to assemble into a rosette and subsequently into a functional KV with a central lumen? to test this idea, we compared recoil velocity and relaxation time of cell interfaces during KV morphogenesis as KV cells transition from non-polarized, to a polarized rosette, to epithelial cells surrounding a fluid filled lumen. We calculated an average recoil velocity of 0.0022 µm/msec for non-polarized pre-rosette KV cells, 0.0075 µm/msec at cell-cell interfaces within a rosette, and then 0.0091 µm/msec post lumen formation, these studies suggest that cell-cell interfaces are supporting increasing amounts of tension as KV lumen forms. We next correlated recoil velocities following laser ablation that were obtained in *in vitro*cell culture of cytokinetic cells at proximal locations to the cytokinetic midbody, a proteinaceous structure located at the center of the cytokinetic bridge. We compared these recoil velocities to KV rosette cell-cell interfaces in proximity to the center of the rosette where cytokinetic bridges are located. With these studies, membrane recoil velocities were greatest closest to the cytokinetic bridge and decreased significantly as targeted ablation was moved away from the cytokinetic bridge. This study suggests that there might be a range in tensions supported along the cytokinetic bridge that is highest next to the cytokinetic midbody and lowest at the furthest point away from the midbody. This creates a testable model in which cytokinetic bridges may be functionally required for packing cells in an energetically unfavorable yet biologically useful confirmation.

P206/B212

Pp2a Inhibitory Protein Sds23 Contributes to Cell Division Symmetry.

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Animal and fungal cells divide through the assembly, anchoring, and constriction of a contractile actomyosin ring (CAR) during cytokinesis. Timing and position of the CAR must be tightly coordinated with events in mitosis, such as spindle assembly and elongation, to prevent defects during the division of the cell. A major regulator of mitotic timing is the conserved heterotrimeric Protein Phosphatase 2A (PP2A), which counteracts the activity of the conserved Cyclin-dependent kinase (CDK), the primary driver of cell cycle progression. Although the role of PP2A in mitotic timing has been well-characterized, the role for PP2A in cytokinesis has been less clear. Loss of PP2A in yeast causes defects in cytokinesis, placing PP2A in pathways that regulate the CAR. Here, we report that Sds23, an inhibitor of PP2A family protein phosphatases, promotes the symmetric division of fission yeast cells through spatial control of cytokinesis and spindle assembly. Fission yeast cells undergo symmetric divisions through medial assembly of the CAR, which results in two equally sized daughter cells following constriction. We found that *sds23Δ* cells divide asymmetrically due to misplaced CAR assembly, followed by sliding of the CAR away from its assembly site. A microscopy-based screen revealed that these mutant cells exhibit delayed recruitment of putative CAR anchoring proteins, including the glucan synthase Bgs1. These results identify the PP2A regulatory network as a critical component in the signaling pathways coordinating cytokinesis. In addition to this role in cytokinesis, we find that Sds23 promotes proper spindle assembly and elongation, as *sds23Δ* cells exhibit asymmetric spindles and decreased rates of spindle elongation. During anaphase in *sds23Δ* cells, the spindle initially elongates asymmetrically toward one end of the dividing cell. This spindle defect is corrected later in anaphase before the CAR constricts. Spindle asymmetry in *sds23Δ* cells results from a novel mechanism that is distinct from previously identified mechanisms including microtubule protrusions. Our results show how Sds23, an inhibitor of PP2A-family protein phosphatases, promotes both bipolar spindle elongation and cytokinesis positioning during fission yeast cell division. These two steps ensure the overall symmetry and success of the cell division process.

P207/B213

The Cell Polarity Kinase Pak1 Controls Cytokinesis and Cell Separation.

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For polarized growth of cells, protein kinases regulate the assembly of cytoskeletal structures at the correct time and place. Large cytoskeletal structures must also be assembled in a regulated manner during cytokinesis and cell separation. We performed a visual screen to identify cell polarity protein kinases that might also function in cytokinesis and cell separation. We found that the Cdc42-activated protein kinase Pak1 (also called Orb2 and Shk1) colocalizes with the assembling cytokinetic ring and remains in the cell middle during septation. Mutations in *pak1* led to defects in cytokinetic ring assembly and cell separation, as well as previously known cell polarity defects. We performed a phosphoproteomic screen and identified novel Pak1 targets that were verified as direct substrates in vitro. Top targets included proteins that function in polarized growth, cytokinesis, and septation. For cytokinesis, we found that Pak1 regulates the localization of its substrates Mid1 and Cdc15 to the cell cortex and the cytokinetic ring. For cell separation, Pak1 phosphorylates the RNA-binding protein Sts5 to prevent its assembly into P-body granules. The cell separation defect of *pak1* mutants was suppressed

by loss of *sts5*. These results show that Pak1 acts directly on components of the cytokinetic ring, but unexpectedly promotes the later stages of cell division by inhibiting the assembly of ribonucleoprotein granules. More broadly, our work reveals that cell polarity signaling proteins coordinate diverse events to promote cell division at the end of the cell cycle.

P208/B214

Microenvironmental Stiffness Promotes Multinucleation Upon Induction of Epithelial-mesenchymal Transition.

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Cytokinetic abscission, the final stage of cell division during which the parent cell physically separates to yield two identical daughter cells, is a complex process. Despite being highly regulated, cytokinesis can fail, generating multinucleated cells. Multinucleation is a sign of genomic instability, an enabling characteristic of cancer. We recently found that the induction of epithelial-mesenchymal transition (EMT), which increases the invasiveness of cells, causes multinucleation in mammary epithelial cells on stiff microenvironments that have mechanical properties similar to those found in breast tumors, but not on soft microenvironments reminiscent of the normal mammary gland. We found that on stiff microenvironments, EMT signaling through Snail upregulates the expression of the midbody-associated filament-forming GTPase, septin-6, and disrupts cytokinetic abscission, thus causing multinucleation. Cells cultured on soft microenvironments do not upregulate septin-6, fail to undergo EMT, and complete cytokinesis normally. Using a septin-6-promoter luciferase-reporter construct, we found that inducing EMT by treating cells with TGFβ or expressing Snail ectopically increases activation of the septin-6 promoter on stiff microenvironments. These data suggest that transcriptional responses to EMT inducers depend on mechanical properties of the microenvironment.

P209/B215

Linear F-actin Promotes Clustering of Rhoa at the Equatorial Plasma Membrane during Cytokinesis.

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During cytokinesis the mother cell is physically divided into two daughter cells. Failure in cytokinesis results in tetraploid cells with supernumerary centrosomes that can cause developmental defects. Constriction of the mother cell is mediated by a contractile ring that assembles underneath the plasma membrane during anaphase. Formation of the contractile ring is triggered by the activation of the small GTPase RhoA, which in turn induces linear F-actin polymerization and myosin II activation. RhoA is prenylated at its C-terminus and is therefore thought to rapidly diffuse within the plasma membrane. How rapidly diffusing active RhoA is maintained within a narrow equatorial zone is less clear due to the difficulty in generating functional fluorescently labeled RhoA probes. To overcome this difficulty we established a RNAi-resistant GFP-tagged RhoA transgene in the nematode *C. elegans* that can replace endogenous RhoA during cytokinesis. We started to investigate GFP::RhoA dynamics in the absence of endogenous RhoA by live-cell imaging one-cell embryos. GFP::RhoA localizes to a narrow equatorial zone and clusters in a dense network during contractile ring assembly. We found that RhoA clusters specifically contain active RhoA and that partial depletion of the RhoA GTPase activating proteins RGA-3/4 broadens the RhoA zone. Therefore, our findings suggest that the formation of a narrow RhoA zone requires rapid flux through the GTPase cycle, a model proposed by Bement and colleagues previously

(Bement et al., 2006). RhoA has two putative membrane targeting motifs: a poly-basic sequence (PBS) and CAAX motif. Our data suggest that the PBS and the CAAX motif are both required for RhoA membrane binding and function however that they are not sufficient to target RhoA to equatorial clusters. To identify factors targeting RhoA to equatorial clusters we tested whether contractile ring components such as linear F-actin has any role. Depletion of the formin CYK-1 did not prevent the accumulation of RhoA but abolished the formation of a dense RhoA network at the cell equator during anaphase. Furthermore in *cyk-1(RNAi)* embryos GFP::RhoA accumulation was delayed and the RhoA zone was broader. In summary, we find that RhoA clusters in a dense network at the equatorial membrane during anaphase. The formation of a narrow RhoA zone depends on formin-nucleated F-actin and rapid GTPase flux through the GTPase cycle. Furthermore formin-induced F-actin promotes the formation of a dense RhoA network at the furrow site. We propose that the formation of a dense RhoA network facilitates contractile ring assembly and successful cytokinesis.

P210/B216

Exclusion of NuMA from the Equatorial Cortex Promotes Timely Cytokinesis.

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NuMA is an essential mitotic protein which guides spindle elongation during anaphase because of its cortical localization and its ability to interact with cortical dynein. In anaphase cortical NuMA localization is dependent on its interaction with phosphoinositides- PtIns(4)P and PtIns(4,5)P2. Interestingly, despite the uniform presence of PtIns(4)P and PtIns(4,5)P2 at the cell cortex, NuMA is excluded from the equatorial cortex during anaphase. We have shown earlier that NuMA is excluded from the equatorial cortical region in a CYK4 dependent manner. However, the mechanisms by which CYK4 excludes NuMA remained elusive. By conducting a candidate-based RNAi screen, we uncovered that beside CYK4, loss of RhoGEF ECT2 that acts downstream of CYK4 also leads to accumulation of NuMA at the equatorial cortex. In the absence of ECT2, NuMA, dynein and dynactin occupy equatorial cortical surface. Therefore, we wondered if the presence of dynein at the equatorial cortical region perturbs robust spindle elongation by counteracting forces from the polar cortical region. Surprisingly, loss of ECT2 marginally impact chromosomes segregation and thus spindle elongation in anaphase. Next, we sought to investigate the mechanism by which ECT2 excludes NuMA from the equatorial cortical region. ECT2 interacts with the similar phosphoinositides to which NuMA binds. To test in vivo if the competition between ECT2 and NuMA for the similar phosphoinositides is responsible for NuMA exclusion, we generated stable cell lines expressing full-length ECT2 and the one lacking lipid-binding potential. Notably, loss of lipid-binding ability of ECT2 cannot prevent NuMA localization at the equatorial cortical region in contrast to the cells expressing full-length ECT2. This data strongly supports the notion that NuMA and ECT2 compete for similar lipids. In a converse experiment, we have artificially targeted NuMA and analyzed its impact on cortical ECT2 and its downstream effector Rho. Importantly, targetting NuMA at the equatorial cortical surface strongly impact Rho levels and significantly delays the furrow initiation. Altogether, our work suggests that cortical NuMA localization in anaphase is spatiotemporally maintained by the RHO-GEF ECT2 and this is vital for regulating proper timing of cytokinetic furrow onset.

P211/B217

Astral Microtubules Regulate Rho Activity and Actomyosin Ring Constriction in Fission Yeast.

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Animal, fungal, and amoeboid cells use microtubules in a mitotic spindle to segregate DNA and an actomyosin contractile ring to form a cleavage furrow during cytokinesis. These different cytoskeletal components must be coordinated to produce viable daughter cells. Work in metazoans has implicated Rho signaling in this integration, though mechanistic details remain incompletely understood. While active Rho is known to localize to the division site in yeast models, evidence for microtubule involvement in its regulation has been lacking. By carefully examining a 20-year-old temperature-sensitive *Schizosaccharomyces pombe* (fission yeast) strain, we discovered evidence for Rho signaling mediating crosstalk between mitotic microtubules and cytokinesis in this organism. *The S. pombe cps1-191* strain was reported to arrest with non-constricting rings at 36°C. Our detailed examination has revealed two insights: 1) rings do not arrest but constrict 30-fold slower than in wild-type cells; and 2) the D277N substitution in β-glucan synthase 1 (Bgs1) that causes temperature sensitivity in the *cps1-191* strain is insufficient for the constriction phenotype. Rings in *bgs1-D277N* cells constrict slower than rings in wild-type cells but faster than rings in *cps1-191* cells. Using whole-genome sequencing, we discovered >250 mutations in *cps1-191*. We further investigated one of these mutations, the S338N substitution in Mto2, a γ-tubulin regulator. Rings constrict as slowly in *bgs1-D277N* cells bearing the *mto2-S338N* mutation or *mto1Δ* (Mto2's binding partner) as in the *cps1-191* strain. However, *mto2-S338N* or *mto1Δ* cells expressing *bgs1*$^{+}$ divide normally, suggesting the Mto1/2 complex plays a redundant role in ring constriction. Others have reported that *mto1Δ* cells lack astral microtubules, which we confirm, and we found that *mto2-S338N* cells also nucleate fewer astral microtubules than wild-type cells. Based on work in other systems, we hypothesized that astral microtubules could influence Rho signaling, which subsequently regulates actomyosin activity. Compared with wild-type cells both *mto2-S338N* and *mto1Δ* cells recruited more Rho1-GTP to the equator, where it persisted longer during cytokinesis. Therefore, we propose that the γ-tubulin complex and astral microtubules have roles in coordinating Rho activity and actomyosin ring constriction in *S. pombe*.

P212/B218

The Integrated Mitotic Stem Cell, a Holistic View of Human Cell Division.

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The Allen Institute for Cell Science is developing a state space of structural signatures of the undifferentiated human induced pluripotent stem cell (hiPSC) to understand the principles by which cells reorganize as they traverse the cell cycle and differentiate. To do this, we take advantage of the fluorescently tagged hiPSC lines that make up the Allen Cell Collection (allencell.org), each expressing an endogenously tagged protein representing a particular organelle or structure. We also develop image-based assays and segmentation algorithms for quantitative analyses, taking advantage of high replicate, high resolution 3D images, which are available online. We have categorized cells in this image collection as mitotic or non-mitotic and sorted the mitotic cells into four categories: prophase, early prometaphase, prometaphase/metaphase, and anaphase/telophase/cytokinesis. From these categorized cells, we created an integrated mitotic stem cell model (imsc.allencell.org) by aligning and overlaying one representative cell for each of five stages (four mitotic plus interphase) of mitosis for 15 structures. We found that all structures could be assigned to three classes of behavior:

redistribution/reorganization, recompartmentalization, and structural maintenance. The integrated model also highlighted relationships between some of the structures, which we further confirmed using "label-free" based structure integration and dual-edited cell line (containing both mEGFP- and mTagRFP-T-tagged structures) approaches. We investigated the variation in structure localization patterns throughout mitosis and found that both cell shape and structure localization patterns were less varied (most stereotyped) during metaphase than in any other stage of the cell cycle. We also found that all reorganizing structures began their transformations in early prometaphase. To identify the order of events during this period of cytoplasmic reorganization, we are sub-dividing the prophase to metaphase transition to create a pseudo-time axis along which to analyze the behavior of reorganizing structures in an integrated fashion. We are also systematically quantifying the variations in organelle inheritance as the cells transition from mitosis into early G1. These observations highlight the usefulness of high-replicate data in understanding structural relationships and changes during cell state transitions.

P213/B219

Stability of the Fission Yeast Contractile Ring Requires Rapid Myo2 and Myp2 Turnover and Lateral an choring of Myo2.

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Actomyosin contractile machines in the cell are intrinsically vulnerable to contractile instabilities: one location that has a little excess contractile material tends to draw in more contractile material, enhancing its contractility so more material is drawn in, and so on, in a runaway process. Here we studied how such instabilities are kept under control in the cytokinetic contractile ring. We mathematically modeled the fission yeast contractile ring incorporating the two myosin-II isoforms found in the ring, Myo2 and Myp2.We find that a stable, homogeneous ring requires that: (i) turnover of Myo2 and Myp2 be faster than a critical value, and (ii) motion of Myo2 and Myp2 in the ring is sufficiently restrained by lateral anchoring to the membrane or crowding effects in the ring, so that the mobilities are below a critical value. If these conditions are not met, the contractile ring is catastrophically affected by a hierarchical density aggregation and tension loss. The contractile ring model is a highly coarse-grained continuum description that incorporates the two myosin-II isoforms: membrane-anchored Myo2 in protein complexes called nodes, and clusters of Myp2 assumed unanchored.The model articulates mechanisms with a clarity not available from more detailed simulation approaches. We find that a runaway buildup of the contractile components, Myo2 and Myp2, is prevented by sufficiently rapid turnover and sufficiently low mobilities. The role of turnover was previously revealed by experiments using cell ghosts of the fission yeast S. japonicus, where turnover is almost completely abolished (Chew et al., 2017). To further explore the effect of turnover on ring stability we developed a complementary approach to this coarse-grained continuum model, a 3D molecularly explicit simulation of rings in cell ghosts with turnover absent. The experiments showed myosin-II puncta moving bidirectionally around the ring and merging into larger puncta. Simulation results showed similar hierarchical aggregation of myosin-II clusters, consistent with these experiments and with our continuum model. These results support the hypothesis that a major role of turnover in the contractile ring is to prevent aggregation instabilities.

P214/B220

Mechanical Feedback Regulates Contractile Ring Constriction and Cell Wall Growth in Fission Yeast and Is Destabilized by Mutations.

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The actomyosin contractile ring constricts and divides the cell during cytokinesis, but is far from isolated. Ring constriction is tightly coupled to membrane addition, cortical and cytoplasmic flow and, in cell-wall enclosed organisms such as fission yeast, septation, the growth of new cell wall. Ring constriction and these coupled processes are highly interdependent, but the mechanisms are poorly understood. In fission yeast, this interdependence is well documented. Mutations of myosin-II in the ring produce irregularly shaped septa and inhomogeneous distributions of beta glucan synthases (Bgs) that grow cell wall (Zhou et al., 2015), while mutations in cell-wall-synthesizing proteins generate misshapen septa and rings, and ring-septum separation (Munoz et al., 2013). These findings are unexplained. Here we developed a molecularly explicit quantitative model that fully integrates the fission yeast contractile ring dynamics and cell wall growth kinetics for the first time. We find the observed irregularities originate in a mechanical feedback system that is destabilized by mutations. In wild type cells mechanical communication between the contractile ring and septum growth systems regulates constriction-septation, but mutations generate positive mechanical feedback that destabilizes both interdependent systems. The 3D ring model incorporates extensive data on component amounts and organization for fission yeast, coupled to a 3D description of stochastic Bgs-mediated septum growth behind the constricting ring, with Bgs mechanosensitivity as quantified (Thiyagarajan et al, 2015). Simulations revealed a symbiotic relation between the ring and septation systems. Ring organization depended profoundly on septum shape because ring tension pulled components to the septum tip, while irregular septum edges drastically impaired ring organization by provoking snapping away of straight bridges. Septum growth depended on the ring, as ring tension set the ring's location and the local septum growth rate. In wild type simulations of the model, the interdependence produced well-organized rings and almost circular septum holes. Ring tension suppressed septum roughness by promoting (suppressing) growth in septum valleys (peaks) while circular septa mechanically regulated ring organization. With mutated components, simulations produced defective organization matching experimental phenotypes. Simulations with mutant *myo2-E1* reproduced the observed straight bridges, non-uniform myosin-II distribution, misshapen septa and irregular septum growth rates correlated with myosin-II distribution (Laplante et al., 2015, Zhou et al., 2015). With mutated Bgs, rings detached into bridges and septa were misshapen as seen experimentally (Munoz et al., 2013).

P215/B221

Laser Ablation Uncovers the Mechanical Properties of the Constricting Contractile Ring in Fission Yeast.

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Cytokinesis in animals, fungi, and amoebas is a robust process that requires the constriction of a contractile ring of actin, myosin, and other conserved proteins. Recent studies have begun to unravel the molecular architecture of the contractile ring. However, we still do not understand how this organization, and its dynamics, support force generation due to the experimental challenges of probing

force in live cells. We overcome this challenge by using laser ablation to cut the contractile ring and measure the kinetics of the resulting severed ends. After cutting the contractile ring, the free ends recoiled away from each other and moved back toward each other to heal the severed ring. The profile of recoil followed an exponential relaxation with interruptions when the recoiling ends stalled for short periods of ~10 s. These data support that severing the contractile ring releases internal tension and that the profile of recoil of the severed ends is influenced by drag forces caused by anchoring of the contractile ring to the plasma membrane/cell wall. We hypothesized that defects in tension production and ring anchoring would alter the kinetics of the severed ends of the contractile ring in response to laser ablation. We cut contractile rings in control, tension-defective (*Δmyp2 myo2-E1*), and anchoring-defective (Cdc15p depleted) cells and found distinct responses in each background. In control cells, the cut free ends of the contractile ring recoiled by ~720 nm over the first 50 s after ablation, after which the recoiling stopped and the cut ring healed over time. In tension defective cells, no recoil was observed after laser ablation and the cut ring healed rapidly. In anchoring defective cells, the cut ring continuously recoiled over the entire imaging period. We tracked these severed rings for ~500 s, until the signal was completely photobleached or the severed ends of the contractile ring left the imaging plane. By the end of our imaging, the severed ends had recoiled ~ 950 nm and healing of the ring was never observed. In conclusion, laser ablation is a powerful tool that can be used to uncover the mechanical properties of the contractile ring and ascribe mechanical functions to cytokinetic proteins.

P216/B222

The an illin Homolog Mid1p Is Dispensable for Cytokinetic Node Assembly in Fission Yeast.

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The contractile ring is a machine built of actin, myosin, and other highly conserved proteins that constricts during cytokinesis. In fission yeast, nodes, protein complexes containing the an illin homolog Mid1p and the Myosin-II molecule Myo2, assemble in a broad band at the site of cell division and coalesce into a contractile ring. Mid1p is thought to be essential for node assembly because broad bands of nodes are not detected in *Δmid1* cells. Rather, strands made of the same cytokinetic proteins as nodes loop to form the contractile ring in these cells. Mid1p leaves the contractile ring prior to the onset of constriction giving rise to the assumption that the node structure is not maintained during constriction. How proteins organize within the constricting contractile ring remains unclear mainly because of the limited tools to probe molecular organization in live cells. We used quantitative high-speed Fluorescence PhotoActivation Localization Microscopy (hsFPALM) in live cells to probe protein organization at the nanometer scale, a ~tenfold improvement in resolution over confocal microscopy. We hypothesized that contractile rings and strands contain nodes that are packed too densely to be resolved by confocal microscopy. Using hsFPALM, we found that both the strands and contractile rings of *Δmid1* cells contain nodes. We found that rings assemble from two different types of strands in *Δmid1* cells: pre-existing and *de novo* strands. Pre-existing strands are present in cells that have previously failed at cytokinesis, can form contractile rings independently of the cell cycle stage, and require actin polymerization by the formin For3p. *De novo* strands assemble as expected during anaphase and are independent of For3p. The molecular organization of nodes in the contractile rings of *Δmid1 and* wild-type cells and in the broad bands of wild-type cells is comparable. However, the distribution of Myo2 heads is more compact in the nodes of pre-existing strands in *Δmid1* cells. This difference in the spreading of the Myo2p heads is actin-dependent. In conclusion, our work 1) shows

that Mid1p is dispensable for node formation and maintenance, 2) indicates that nodes are components of the contractile ring, and 3) suggests that node protein organization is governed by the molecular composition and organization of the underlying actin network.

P217/B223

An Unexpected Twist to Escrt Proteins.

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The ESCRT-III is a conserved protein complex that mediates membrane remodeling and scission in the context of essential cellular processes among which multi-vesicular body formation, cell division and virus budding. The ESCRT pathway has been extensively studied in *vivo and* reconstituted in *vitro* using yeast proteins (Snf7, Vps2, Vps24). In *Homo Sapiens*, the ESCRT-III complex is composed of at least 12 proteins, called Charged Multivesicular Body Protein (CHMP 1-7). We have focused our attention on CHMP4 (human homolog of yeast Snf7), CHMP2 and CHMP3 (homologs of yeast Vps2 and Vps24 respectively). It has been proposed that filaments composed of Snf7/CMHP4, the main structural component of ESCRT-III polymer, can form spiral springs that would promote membrane deformation by buckling (Chiaruttini et al.). To address whether ESCRT proteins can remodel membranes, we have used a bottom-up approach and analyzed, using cryo-electron microscopy and cryo-tomography, how different combinations of ESCRT proteins would deform lipid vesicles, in vitro. When bound to biomimetic membranes, spirals of CHMP4 are flat and membranes are not significantly deformed. Instead, the spirals would rather have a tendency to flatten liposomes. Unexpectedly, the addition of a combination of CHMP2A and CHMP3 or CHMP2B together with CHMP4 induced a dramatic tubulation of vesicles. Most of the membrane was remodeled and tubulated. Regular “Corskrew-like” or “zigzag”-like tubular patterns were visible in 2D images or 3D reconstructions obtained from cryo-tomography. Besides two sets of filaments were visualized, one being parallel to the axis of the tubes and the other one being orthogonal. Hence, CHMP4, by itself, was unable to deform membranes, in *vitro* and required the presence of CHMP2/3 proteins. We believe that this behavior might reflect the activity of ESCRT proteins in membrane remodeling in situ. **References** CHIARUTTINI, N et al., 2015. Relaxation of Loaded ESCRT-III Spiral Springs Drives Membrane Deformation. Cell, 163(4), pp. 866-879.

P218/B224

Cytokinesis in *Silico*: Development of an Agent-based Model of Actomyosin Behavior in the Contractile Ring of an Early Embryo.

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We have used super-resolution light microscopy and platinum replica TEM to elucidate the ultrastructural organization of the contractile ring (CR) in early sea urchin embryos. These experiments employ an isolated cortex approach that takes advantage of the large size of the embryo’s CR (10X larger than a cultured mammalian cell), the thinness of the preparation (allowing for TIRF/SIM microscopy and platinum replica TEM), and the unique perspective afforded by examining the CR on the cytoplasmic face of the plasma membrane. To date our studies suggest that the CR initiates as a broad stripe of clusters containing myosin II, actin, septin2 and anillin, which then appear to congress over time into the narrower, linear array characteristic of mature CRs. In parallel with these imaging

experiments, we are examining the behaviors of CR-related actomyosin assemblages in *silico*. Our 3D agent-based computer model incorporates detailed characteristics of actin and myosin II that include: 1) actin filament polymerization and monomer hydrolysis status, filament flexibility, severing, crosslinking, and interaction with myosin II heads; 2) myosin II motors that are approximated as three rigid physics bodies —head, neck and tail— constrained by springs that undergo ATPase-based changes in conformation to generate force via power strokes. Myosin and actin filament behaviors are benchmarked with known biophysical properties and classic in *vitro* assays. Our preliminary simulations include the generation of CR-like organization in an *S. pombe*-based context, where myosins move larger protein nodes along actin filaments. In addition, we are examining higher order emergent behaviors in simulations based on assemblages of anti-parallel actin filaments and myosin II mini-filaments; here dense actomyosin patches form that mirror the coalescence of clusters seen in our imaging of CR dynamics in sea urchin embryos. We are incorporating information derived from our imaging experiments - such as distances between clusters, approximate numbers of myosin filaments per cluster, rate of cluster congression, location of scaffolding proteins, and orientation of actin filaments - into the in *silico* model in order to determine if we can recapitulate the cluster to linear array transition. We are confident that a productive crosstalk between our imaging experiments and these computational simulations will result in a deeper understanding of CR structure and function.

P219/B225

Chromatin Sensing Regulation of Cytokinesis Is Stronger in Cancer Cells with Higher Ploidy.

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Cytokinesis occurs at the end of mitosis and leads to the physical separation of two daughter cells. This process needs to be tightly regulated to prevent aneuploidy and cell fate changes. It is mediated by the ingression of an actomyosin contractile ring that pulls the plasma membrane inwards to separate the two cells. Assembly and ingression of the ring is spatiotemporally regulated by spindle-dependent and -independent pathways. Recently, our lab found that a cue associated with chromatin also regulates cytokinesis by influencing the function of anillin, which is a component of the ring. During interphase, proteins with nuclear localization signals (NLS) are bound by importins and brought into the nucleus where Ran-GTP releases them. High levels of Ran-GTP are found in the nucleus since its GEF, RCC1, is associated with chromatin, and low levels of Ran-GTP are in the cytosol where Ran GAP is located. During mitosis, this gradient of active Ran is retained with low levels of Ran-GTP near the cortex permitting importins to remain bound to proteins. Our lab found that importins bind to the NLS of anillin to mediate its cortical recruitment and function for cytokinesis. Interestingly, prior studies found that anillin is highly over-expressed in many cancers, and other studies showed that cancer cells with aneuploidy have steeper Ran-GTP gradients. Thus, we propose that cancer cells with aneuploidy rely more heavily on chromatin-sensing and anillin function for cytokinesis. This would ensure that furrow positioning correlates with chromatin position to avoid mitotic catastrophe. To test this, we determined the requirement for anillin in aneuploid cancer cells (A549 and HeLa), compared to near-diploid cancer cells (HCT116) and normal fibroblasts (HFF-1). Following anillin knockdown, HeLa and A549 cells frequently failed cytokinesis, while HCT116 and HFF-1 were significantly less affected. However, these cells originate from different tissues, which could explain their different requirements for anillin. To further determine if ploidy contributes to changes in anillin-dependency, we altered the ploidy of HCT116 cells and determined their requirement for anillin. A higher proportion of HCT116 cells with

higher ploidy failed cytokinesis compared to their lower ploidy counterparts. To continue to test the relationship between ploidy and anillin requirement, we used CRISPR to endogenously tag anillin with mNeonGreen in HCT116 cells. With this tool we can separate cells based on ploidy, then correlate precise changes in anillin levels with cytokinesis phenotypes in cells with low (2n) vs. high (4n or 8n) ploidy. This work sheds light on how the Ran pathway may help aneuploid cells avoid mitotic catastrophe during mitosis, and could be a novel therapeutic target against cancer cells.

P220/B226

Role of the Cdc48 Segregase in the Robust Execution of an aphase.

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Cdc48 (p97/VCP) is a AAA+ ATPase which can separate proteins from their binding partners and unfold them in the process. Cdc48 typically acts on ubiquitinated proteins, which it can bind through its cofactor complex Ufd1/Npl4. By segregating ubiquitinated substrates from their non-ubiquitinated binding partners, Cdc48 may protect the non-ubiquitinated partner from co-degradation. Securin and cyclin B are two key proteins whose degradation is critical in mitotic progression. The degradation of securin liberates its binding partner separase; separase then cleaves cohesin, resulting in sister chromatid separation. Degradation of cyclin B leads to declining Cdk1 activity and mitotic exit events. Interestingly, fission yeast *cdc48* mutants have been shown to contain low levels of separase and lose viability during mitosis. This suggests a role for Cdc48 in mitosis, possibly by acting on ubiquitinated securin. We find lower cellular levels of securin but normal levels of cyclin B in the fission yeast *cdc48* mutant. In addition to the lower securin levels, we observe slower kinetics in the drop of nuclear securin and delayed sister chromatid separation by live cell imaging. In contrast, the rate at which nuclear cyclin B drops and the events downstream of its degradation are unaffected. We also observe slower kinetics in the drop of nuclear securin in a *ufd1* mutant, supporting that Cdc48 may act on ubiquitinated securin. We are investigating the mechanisms by which Cdc48 regulates separase and securin levels, and the factors or features that dictate the specificity of Cdc48 towards the securin-separase arm of mitotic regulation. Overall, our results hint to a pivotal role of Cdc48 for anaphase, whose mechanistic basis remains to be elucidated.

P221/B227

Screening for Deubiquitinating Enzymes an tagonizing APC/C Activity in Fission Yeast.

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The anaphase promoting complex (APC/C) is an E3 ubiquitin ligase which triggers anaphase by ubiquitinating securin and cyclin B, thereby targeting them for proteasomal degradation. While the mechanisms governing APC/C activity have been extensively studied, much less is known about deubiquitinating enzymes (DUBs) that may antagonize APC/C activity. To find antagonists of the APC/C, we overexpressed individual DUBs in fission yeast (*S. pombe*) and tracked the degradation kinetics of GFP-tagged cyclin B and securin by live-cell imaging. We focused on DUBs that have been reported to localize to the nucleus, where the APC/C, securin and cyclin B are enriched during mitosis. We found that overexpression of *S.p.* Ubp16 (*S.c.* Ubp10) lead to elevated securin levels, a slight reduction in the securin degradation kinetics, and appearance of the 'cut' phenotype, where cytokinesis proceeds without chromosome separation. This complements findings by others in budding yeast that overexpression of Ubp10 stabilizes APC/C substrates. Deletion of *ubp16* slightly rescued growth of an

APC/C mutant strain, supporting that Ubp16 may antagonize the APC/C. We are continuing to screen for additional candidates and investigating whether Ubp16 indeed plays a role during mitosis and if it acts directly on securin or other substrates.

P222/B228

Regulations of UNC-13/Munc13 Protein Ync13 Localization and Function during Fission Yeast Cytokinesis.

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Ync13 is a member of the UNC-13/Munc13 protein family, whose animal homologs are essential priming factors for SNARE complex assembly during exocytosis. Our previous studies suggest that Ync13 is involved in both exocytosis and endocytosis. Ync13 is critical for the normal distribution of cell-wall enzymes and some other proteins on the division plane. β-Glucan synthase Bgs4, a transmembrane protein, accumulates at the center instead of its normal even distribution on the division plane in *ync13Δ* cells. However, it remains unknown how Ync13 localization and functions are regulated since its binding partners have not been identified. Here we found that Rho-GAP Rga7 and coiled-coil protein Rng10 are binding partners of Ync13 by affinity purification and mass spectrometry. Rga7, Rng10, and Ync13 colocalized on the plasma membrane at the cell tips during interphase and at the division site during cell division. Ync13 was almost abolished at division site in *rga7Δ* or *rng10Δ* cells. In addition, mistargeted Rga7 or Rng10 by Tom20 could recruit Ync13 to mitochondria, which suggesting that the localization of Ync13 depends on both Rga7 and Rng10. Moreover, mutants of *ync13*, *rga7*, and *rng10* had similar cell-lysis phenotype and were synthetic lethal. We hypothesize that Rga7 and Rng10 help to anchor Ync13 on the plasma membrane at the division site to regulate Bgs4 trafficking during cytokinesis. Consistently, Bgs4 level at the division site is significantly lower in *rga7Δ* and *rng10Δ* cells and moved slowly from the late Golgi compartments to the cleavage site in *rga7Δ* cells. We also found mistargeted Rga7 or Rng10 recruited Bgs4 to mitochondria, indicating that Rga7/Rng10 may directly interact or form a complex with Bgs4. The late arrival and lower levels of Bgs4 result in septal defects in cytokinesis and the lysis of separating cells.

Kinetochore Assembly and Functions 1

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Essentiality of CENP-A Depends on Its Binding Mode to HJURP.

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The centromere is an essential genome region where the kinetochore is established for faithful chromosome segregation. In most organisms, centromeres are specified by sequence-independent epigenetic mechanisms through deposition of histone H3-variant CENP-A into chromatin. Therefore, it is critical to know how CENP-A is deposited into centromeric chromatin. We previously showed that the chicken Mis18 complex directly binds to the CENP-A nucleosome and is recognized by the pre-deposition CENP-A-H4-HJURP (CENP-A specific chaperon) complex for new CENP-A incorporation (Hori et al., Dev. Cell, 2017). But, it is still unknown how the pre-deposition CENP-A-H4-HJURP complex correctly transfers the CENP-A into centromeric chromatin. Here, we analyzed various CENP-A domains

in the chicken DT40 cells, using a gene complementation assay. We found that while both N- and C-terminal tails of CENP-A were dispensable, the alpha-1 helix region near CATD (CENP-A targeting domain) was essential for CENP-A deposition and centromere maintenance in chicken DT40 cells, which is not the case for human CENP-A. Consistent with our CENP-A characterization, a contact residue in HJURP to the CENP-A alpha-1 helix was also essential in DT40 cells. To understand why the CENP-A alpha-1 helix is essential in chicken but not in human, we prepared a chimeric HJURP in which the CATD binding region of chicken HJURP was replaced with the corresponding region of human HJURP. Surprisingly, expression of the chimeric HJURP suppressed CENP-A incorporation defects in cells expressing the CENP-A alpha-1 helix mutant. Additional biochemical analyses revealed that binding affinity of CATD to HJURP was stronger in human than in chicken, suggesting that CATD-HJURP interaction is sufficient and is not necessary to use the CENP-A alpha-1 helix for its HJURP binding in human cells. We also identified key residues in human HJURP sufficient for CENP-A binding in the absence of the alpha-1 helix contact. Interestingly, HJURP in most species except for some of the mammals do not contain these residues and appear to use the CENP-A alpha-1 helix for HJURP binding. We propose that essentiality of CENP-A depends on its binding mode to HJURP, which is variable during evolution. This gives us a new insight into how CENP-A is co-evolved with HJURP.

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CENP-C Unwraps the CENP-A Nucleosome through the H2A C-terminal Tail.

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Centromeres are defined epigenetically by nucleosomes containing the histone H3 variant CENPA, upon which the constitutive centromere-associated network of proteins (CCAN) is built. CENPC, is considered to be a central organizer of the CCAN. We provide new molecular insights into the structure of CENP-A nucleosomes, in isolation and in complex with the CENP-C central region (CENP-C^{CR}), the main CENP-A binding module of CENP-C. We establish that the short αN-helix of CENP-A promotes DNA flexibility at the nucleosome ends, independently of the sequence it wraps. Furthermore, we show that, in *vitro*, two regions of CENP-C (CENP-C^{CR} and CENP-C^{motif}) both bind exclusively to the CENP-A nucleosome. We find CENP-C^{CR} to bind with high affinity due to an extended hydrophobic area made up of CENP-A^{V532} and CENP-A^{V533}. Importantly, we identify two key conformational changes within the CENP-A nucleosome upon CENP-C binding. First, the loose DNA wrapping of CENP-A nucleosomes is further exacerbated, through destabilization of the H2A N-terminal tail. Second, CENP-C^{CR} rigidifies the N-terminal tail of H4 in the conformation favoring $H4^{K20}$ monomethylation, essential for a functional centromere.

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CDK1 Regulates CENP-A-CENP-C Interaction in Vertebrate Kinetochore.

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Chromosomes duplicated in S-phase have to be accurately segregated to daughter cells in M-phase. The accurate chromosome segregation requires the kinetochore, a large protein complex, which makes a linkage between chromosome and spindle microtubules. Clarifying how the kinetochore is assembled is critical for understanding of the principles of the accurate chromosome segregation. The kinetochore is formed on the centromere of each chromosome that is epigenetically defined by CENP-A, a centromere-specific histone H3 variant. Constitutive Centromere Associated Network (CCAN), a kinetochore

subcomplex, localizes to the centromere throughout the cell cycle via binding of CENP-A nucleosome. In M-phase, CCAN recruits another kinetochore subcomplex KMN (Knl1, Mis12 and Ndc80 complexes) network, which directly binds to microtubules, connecting chromosomes to spindle microtubules. As CCAN provides the centromere-kinetochore interface, a key question to be addressed is how CCAN interacts to the centromere and how the interaction is regulated. CENP-C is one of CCAN subunits, which directly binds to the CENP-A nucleosome in vitro. We recently showed that the CENP-C C-terminus region interacts with centromere during M-phase but not during interphase in chicken DT40 cells, suggesting that the CENP-C-centromere interaction is regulated in cell cycle progression, although CENP-C constitutively localizes to centromere. Then, the question we set to clarify was how the dynamic CENP-C-centromere interaction was regulated. We found that a conserved motif, CENP-C motif, which binds to the CENP-A nucleosome, is required for the CENP-C-centromere interaction in M-phase. We also identified a key CDK1 phosphorylation site that regulates the CENP-C-centromere interaction. In fact, mutation of this site reduced centromere localization of CENP-C in M-phase. Further analyses revealed that the CDK1 phosphorylation facilitates interaction between the CENP-C motif and the CENP-A nucleosome. Finally, we demonstrated that the CDK1-depenent CENP-C-centromere interaction contributes to chromosome segregation. These results suggest that CENP-C phosphorylation by CDK1 is a key regulation for the CENP-A-CENP-C interaction in M-phase and provides a model in which dynamic interaction of the centromere-CCAN during cell cycle progression is important for functional kinetochore assembly.

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Constitutive Presence of R Loops at Centromeric Chromatin Contributes to Chromosome Instability.

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Aneuploidy is a hallmark of many cancers and a significant driver of tumorigenesis. An euploidy, observed in 90% of solid tumors, is caused by chromosome instability (CIN), characterized by the unequal distribution of chromosomes into two daughter cells and/or structural rearrangements of the genome. One of the key determinants for chromosomal stability is the centromere, which serves as a site for kinetochore assembly, mediates kinetochore-microtubule attachment and spindle assembly checkpoint function. R-loops, which are the by-product of DNA-RNA hybridization, are largely undetected at the centromeric chromatin in budding yeast and reported to persist in a *HPR1* deletion strain. However, the molecular role of R loops at the centromeres and its physiological significance remain poorly understood. Here we report that constitutive presence of centromeric R loops affects kinetochore integrity and CIN in budding yeast. We show that Hpr1, associates with the centromeric chromatin, and interacts in *vivo* with the evolutionarily conserved centromeric histone H3 variant Cse4 (CENP-A in humans). Deletion of *HPR1* causes accumulation of centromeric R loops, reduction in the levels of centromere-bound Cse4 and its assembly factor Scm3 (HJURP in humans), with a concomitant increase in histone H3 at the centromeric chromatin. We found that constitutive presence of centromeric R loops interferes with kinetochore function as increased frequency of chromosome loss were observed *hpr1Δ* strains. In summary, our results have begun to uncover the mechanisms by which constitutive presence of centromeric R loops contributes to CIN and may contribute to aneuploidy in cancers.

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The Role of Rad50 in Cenp-a Deposition at the Centromere.

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In most eukaryotes, the centromere is a single chromosomal locus that attaches to microtubules to ensure delivery of one copy of each pair of sister chromatids to daughter cells during cell division. Centromere identity does not rely on specific DNA sequences, but, rather, is associated with large arrays of repetitive DNA. Centromere specification is thought to occur via deposition of the centromere-specific histone H3 variant, CENP-A. After DNA replication, "old" centromeric nucleosomes are distributed to the replicated chromatids. In mammals, deposition of newly synthesized CENP-A occurs at the centromere in the G1 phase of the cell cycle. This step is crucial for proper centromere inheritance and function. Yet the molecular mechanism remains unclear. We have shown that CENP-A deposition at the centromere requires ubiquitylation on lysine 124 (K124) that is catalyzed by the CUL4A-RBX1-COPS8 E3 ligase. Introduction of the K124R mutation abrogates the centromeric localization of CENP-A, while addition of a mono-ubiquitin at the C-terminus of CENP-A K124R restores its centromeric localization (*Dev Cell, 2015*). Fachinetti et al. reported that EYFP-CENP-A K124R mutant cells are viable (*Dev Cell, 2017*). However, we recently found that EYFP tagging, as used in Fachinetti et al., induces a non-physiological ubiquitylation event at a different lysine of EYFP-CENP-A-K124R, which allows EYFP-CENP-A-K124R to bind to HJURP. We also found that Flag-tagged or untagged CENP-A K124R mutants are nonfunctional in cells (*Dev Cell, in press)*. We reported that overexpression of mono-ubiquitin-fused CENP-A creates ectopic centromeres at non-centromeric regions (neocentromeres) (*Cell Reports, 2016*), suggesting that identifying ubiquitin-dependent CENP-A interactors provides crucial information regarding the mechanisms that control centromere assembly and maintenance. Our IP-mass spec has identified RAD50 as a ubiquitin-dependent CENP-A interactor. IP-Western blots confirmed that CENP-A interacts with the MRN complex (RAD50, MRE11, and NBS1). We found that RAD50 depletion delocalizes CENP-A from the centromere, suggesting that the MRN complex is required for CENP-A deposition at the centromere. The MRN complex is structurally similar to SMC complexes; both bind to CENP-A, and both are required for CENP-A deposition. This finding implies that the MRN complex may stabilize CENP-A in nucleosomes. We also found that DHX9 (RNA helicase A) binds CENP-A in a CENP-A ubiquitylation-dependent manner. DHX9 is involved in R-loop (which contains DNA-RNA hybrids and displaced ssDNA) formation or resolution. ATR is activated by R-loops at the centromere for faithful chromosome segregation. These results suggest that CENP-A is involved in ATR activation via R-loops at the centromere.

P228/B234

Cenp-a Overexpression and Mislocalization Contribute to an euploidy and Karyotypic Heterogeniety in Human Cells and Xenograft Mouse Model.

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Aneuploidy results from chromosomal instability (CIN) and contributes to tumorigenesis and tumor heterogeneity. Therefore, it is crucial to understand the molecular pathways contributing to aneuploidy. Centromere associated proteins are the key regulators for chromosomal stability. Centromeric

localization of an evolutionarily conserved histone H3 variant, CENP-A is essential for chromosomal stability. Overexpression (OE) and mislocalization of CENP-A to non-centromeric regions contributes to CIN in yeast and flies. CENP-A OE and mislocalization have been observed in several cancers and correlates with poor prognosis. However, the correlation between CENP-A mislocalization, aneuploidy and tumorigenesis has not been investigated. We recently showed that mislocalization of CENP-A to non-centromeric regions results in CIN in HeLa and RPE1 cells. Here, we show that CENP-A mislocalization contributes to aneuploidy and enhances the tumorigenic potential of DLD1 cells with CENP-A OE in doxycycline inducible manner ($DLD1^{Dox\text{-}CENP\text{-}A\text{-}YFP}$). CENP-A mislocalization to non-centromeric regions contributes to CIN in $DLD1^{Dox\text{-}CENP\text{-}A\text{-}YFP}$ cells, as ascertained by defective chromosome segregation and increased incidence of micronuclei. Mislocalization of CENP-A weakens the kinetochores due to reduced levels of outer kinetochore protein, Nuf2, creating unstable kinetochore-microtubules attachments in $DLD1^{Dox\text{-}CENP\text{-}A\text{-}YFP}$ cells. To investigate if the mislocalization of CENP-A affects the metastatic and invasiveness of cells, we performed trans-well assays. Higher invasion, that could be rescued by suppression of CENP-A mislocalization, was observed in $DLD1^{Dox\text{-}CENP\text{-}A\text{-}YFP}$ cells, but not in control DLD1 cells, confirming that CENP-A mislocalization enhances the invasiveness of cells. To examine whether CIN phenotypes we observed in cultured cells could be translated *in-vivo*, we used a xenograft mouse model using $DLD1^{Dox\text{-}CENP\text{-}A\text{-}YFP}$ cells. Tumors in mice fed with DOX diet regressed significantly at first, followed by slow growth. Cells derived from these tumors displayed CENP-A mislocalization, higher incidence of micronuclei and severe aneuploidy with karyotypic heterogeneity. Consistent with the slow growth of tumors, prolonged treatment of $DLD1^{Dox\text{-}CENP\text{-}A\text{-}YFP}$ cells with high concentration of DOX (1.0µg/ml for 5 days) showed positive staining for apoptosis marker, an nexinV in the majority of cells. These results suggest that even though CENP-A induced aneuploidy leads to apoptosis and slow growth of tumors, the karyotypic heterogeneity may contribute to poor prognosis of cancers with CENP-A OE. In summary, our results show that CENP-A OE and mislocalization contribute to aneuploidy and karyotypic heterogeneity in cell lines and xenograft mouse model.

P229/B235

Centromere Incompatibility as the Basis for Chromosome Segregation Defects in Inviable *Xenopus*Hybrids.

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Centromere incompatibility as the basis of chromosome segregation defects in inviable *Xenopus* hybrids Maiko Kitaoka* and Rebecca Heald Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, USA *Presenting author: mkitaoka@berkeley.edu **ABSTRACT** Despite the evolutionarily conserved function and importance of centromeres to mediate faithful segregation of replicated chromosomes, the underlying DNA sequences and centromeric proteins are rapidly evolving, even among closely related species. Interestingly, faulty chromosome segregation has been reported in a number of inviable hybrids, contributing to post-zygotic developmental barriers and speciation. However, the molecular mechanisms underlying specific chromosome loss in hybrids is poorly understood. *Xenopus* frog species provide an ideal system to study hybridization and genome evolution, as hybrids produced when *X. laevis* or *X. borealis* eggs are fertilized by *X. tropicalis* sperm (*l*e×*t*s and *b*e×*t*s) are viable, but the reverse crosses (*t*e×*l*s and *t*e×*b*s) are not. Both inviable hybrids show chromosome segregation defects, however, whole genome sequencing revealed that *t*e×*l*s hybrids lose two specific *X. laevis* chromosomes, while *t*e×*b*s hybrids lose regions of four specific *X. borealis* chromosomes. We used *Xenopus* egg extracts to demonstrate that a subset of *X. laevis* and *X. borealis*

chromosomes lost CENP-A, the core centromeric histone variant, as well as Ndc80, the key outer kinetochore component that attaches to spindle microtubules, after one cell cycle. CENP-A loss is cell cycle-dependent, as unreplicated paternal sperm retain all CENP-A localization. *X. laevis* chromosome centromere localization is rescued with addition of *in vitro* transcribed and translated CENP-A with its chaperone HJURP to *X. tropicalis* cytoplasmic extracts, although *X. borealis* centromeres are not rescued. Preliminary results suggest that transcription affects centromere assembly in these hybrids, as triptolide inhibition of RNA Pol II can also rescue CENP-A localization. Ongoing experiments aim to rescue segregation defects in inviable hybrid embryos and uncover the mechanisms underlying specific paternal chromosome mis-segregation and inviability in *Xenopus* hybrids.

P230/B236

Centromere Strength Is Transgenerationally Inherited through the Male but Not Female Germline.

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Centromeres direct genetic inheritance but are not themselves genetically inherited. Instead, centromeres are defined epigenetically by the presence of a histone H3 variant, CENP-A. According to existing models for centromere inheritance, preexisting CENP-A nucleosomes serve as templates to direct new assembly, which quantitatively maintains centromere chromatin through every cell cycle in cycling somatic cells. To test this model in vivo in the mammalian germline, we created CENP-A hemizygous mice with reduced levels of centromere chromatin in the gametes. We show that genetically wild-type progeny from two *cenpa+/-* hemizygous parents have partially reduced centromere chromatin in somatic tissue and in the male germline and that these levels are transgenerationally inherited. But we find wild-type levels of CENP-A in the female germline, indicating that CENP-A levels reset prior to prophase I arrest in the oocyte. Furthermore, when only one parent is hemizygous, we don't observe any detectable differences between maternal and paternal centromeres in adult progeny, indicating equilibration between maternal and paternal centromeres. Based on these results, we propose two key exceptions to template-dependent centromere inheritance. First, a unique assembly mechanism in the female germline increases CENP-A chromatin, possibly to protect against loss during the prolonged prophase arrest. Second, CENP-A chromatin can differ quantitatively between sperm and egg chromatin in the zygote, but CENP-A redistributes in the early embryo to equalize maternal and paternal centromeres, providing a mechanism to suppress centromere drive.

P231/B237

Enrichment of Aurora B Kinase at the Inner Kinetochore Controls Outer Kinetochore Assembly.

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The outer kinetochore is assembled and disassembled every cell cycle, and loss of this regulation can lead to errors in chromosome segregation. Aurora B kinase plays a pivotal role in kinetochore assembly through the phosphorylation of the Mis12 complex (Mis12C) subunit Dsn1. Current models posit that Dsn1 phosphorylation relieves autoinhibition, which allows Mis12C binding to the inner kinetochore component CENP-C. However, the mechanism by which kinetochore assembly is restricted to centromeres is unknown. Using *Xenopus* egg extracts and biochemical reconstitution, we show that multiple factors synergize to ensure the specificity of kinetochore assembly. First, we find that centromere-bound CENP-C molecules become static in mitosis and do not exchange with the soluble pool, as observed in human cells. This shift in CENP-C dynamics restricts productive formation of

Mis12C-CENP-C complexes to kinetochore-localized CENP-C. Second, we show that Mis12C autoinhibition by the N-terminal tail of Dsn1 serves as a barrier to phosphorylation by Aurora B kinase. Our data indicate that substrate access is enhanced through transient interactions of the Mis12C with kinetochore-bound CENP-C, and that the Dsn1 phosphorylation rate is enhanced by high local concentrations of Aurora B kinase proximal to CENP-C. We will discuss our latest efforts to identify the receptor for the CPC at inner kinetochores. Thus, we propose that the coincidence of CENP-C and Aurora B at inner kinetochores is required for proper kinetochore assembly only at centromeres.

P232/B238

Building a Functional Kinetochore: from Microtubule to Centromere.

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Perhaps the largest and most intricate of all microtubule-associated protein machines, the kinetochore consists of over fifty different proteins organized into subcomplexes. Their architecture is branched, with more copies of the outer microtubule-binding subcomplexes than centromeric nucleosomes at their base. The kinetochore performs vital mechanical functions during mitosis: coupling chromosome movement to microtubule dynamics and sensing the tension that signals chromosome biorientation. Much is known about the microtubule-coupling interface of the kinetochore, and previous work has demonstrated that the Ndc80 subcomplex binds to microtubules and transmits force to the Mis12 subcomplex (Mis12c^{MIND}). However, it has been unclear how the distinct, underlying branches within the centromere-proximal inner kinetochore contribute to the kinetochore's load-bearing ability. Here, using a set of six recombinantly purified budding yeast kinetochore subcomplexes, we show that they can spontaneous self-assemble into functional, load-bearing tethers between dynamic microtubule tips and nucleosomes containing the centromere-specific histone variant. We quantify the load-bearing and microtubule tip-coupling abilities of these reconstituted kinetochores using an optical trap and demonstrate that there are two distinct and essential paths of force transmission through the inner kinetochore. These two paths, one through CENP-QU^{OA} and the other through CENP-C^{Mif2}, are stronger in combination than alone. Moreover, our work corroborates the finding that the Mis12c^{MIND}-inner kinetochore interface is regulated by the major mitotic kinase, Aurora B, which alleviates autoinhibition within Mis12c^{MIND}. Intriguingly, these two paths of force transmission through the inner kinetochore appear differentially sensitive to Aurora B phosphorylation. Taken together, this work represents a major advance towards the goal of reconstituting a complete, functional kinetochore from purely recombinant proteins.

P233/B239

Set, Pp2a Inhibitor, Fine-tunes Aurora B Kinase Activity at Centromere in a Tension-dependent Manner for Proper Chromosome Segregation.

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Aurora B (AurB) kinase phosphorylates kinetochore substrates to destabilize improper kinetochore-microtubule (KT-MT) attachments, whereas the opposing phosphatase PP2A dephosphorylates these substrates to stabilize correct attachments. Thus, spatiotemporal phosphor-regulation at kinetochore is essential to establish chromosome bi-orientation and prevent aberrant chromosome segregation and aneuploidy, a hallmark of cancer. However, little is known about the molecular mechanism that tunes

the balance of phosphorylation at kinetochore. A proto-oncogene SET, a PP2A inhibitor, is well known to overexpress in many cancers, but the mechanism by which SET overexpression induces malignancy remains largely unknown. Here, we show that SET fine-tunes AurB kinase activity in a distance-dependent manner among sister kinetochores to regulate accurate chromosome alignment. In prometaphase (low tension), SET localized at centromere through direct interaction with Shugoshin2 maintains AurB activity by inhibiting PP2A. However, in metaphase (high tension), SET dissociation from centromere stabilizes bi-oriented attachments by activating PP2A, which results in decrease of AurB activity. These data suggest that a distance-dependent feedback loop between SET, Aurora B and Bub1 functions as a tension sensor at the centromere/kinetochore, and overexpression of SET promotes aberrant chromosome segregation and aneuploidy by inducing dysregulation of AurB. The following results suggest our conclusions. 1. In prometaphase, SET maintains AurB activity at centromere to regulate chromosome alignment. SET depletion or its overexpression, but not the overexpression of the mutant defective for interaction with Shugoshin2 at centromeres, affect AurB activity, and thereby induces chromosome misalignment and aneuploidy. 2. In prometaphase, centromeric pools of both AurB and Shugoshin2/SET are regulated by Bub1 at kinetochores through Bub1-mediated phosphorylation on HH2A at Thr-120. Similarly, AurB regulates Bub1 pools at kinetochore, suggesting a positive feedback loop between the amounts of SET and Bub1, and AurB. 3. In metaphase, SET dissociates from centromeres, depending on decrease of Bub1 pool at kinetochores, which results in stabilization of bi-oriented attachments by activating PP2A and inactivating AurB. Ref.: Y. Asai et al., JCB. (2019), Y. Terada et al., EMBO J. (1998)

P234/B240

A Universal Mechanism Underlying Impaired Centromeric Cohesion in Cancer Cells.

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Centromeric cohesion is essential for proper chromosome segregation. Impaired centromeric cohesion are often found in cancer cells. The defects may contribute to chromosome instability and aneuploidy, which are major hallmarks for cancer. However, the mechanisms that underlie impaired centromeric cohesion in cancer cells are poorly understood. We and others recently showed that SET, a potent phosphatase 2A (PP2A) inhibitor, is also an inhibitor to the essential cohesion protector, Shugoshin. In a screening for SET protein levels in nearly 30 cell lines from various types of cancers, we found that most of the cancer cells exhibited much higher levels of SET than the non-transformed cell RPE-1, which is consistent with the results from various previous studies. Interestingly, we also found that the high levels of SET protein are positively related to centromeric cohesion defects in these cancer cells. Partial knock-down SET protein levels significantly rescued the centromeric cohesion defects for these cancer cells. Thus, highly expressed SET is a major factor that contributes to the impaired centromeric cohesion in cancer cells. As SET is also PP2A inhibitor and PP2A activity is often repressed in cancer cells, we also addressed whether lower PP2A activity is also responsible for the impaired centromeric cohesion in cancer cells. Surprisingly, we found that pharmaceutically increasing PP2A activity in these cancer cells did not ameliorate centromeric cohesion, suggesting that compromised PP2A activity in cancer cells may not be one of the major driving forces for impaired centromeric cohesion. Based on these findings, we propose that highly expressed SET in cancer cells contributes to impaired centromeric cohesion defects mainly through inhibiting Sgo1 functions not through inhibiting PP2A activity. As the phenomenon has been found in many types of cancer cells, we also propose that it is a major and universal mechanism underlying impaired centromeric cohesion in cancer cells.

P235/B241

Separase Cleaves the Kinetochore Protein Meikin to Direct the Meiosis I/II Transition.

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Meiosis is a specialized cell division that reduces the genetic content of a cell by half through two successive rounds of cell division. To achieve a reductional division during meiosis I, sister kinetochores must co-orient to transduce forces to the same spindle pole. In addition, cohesin in the centromeric region must be protected from cleavage by the protease Separase to ensure that sister chromatids remain associated until anaphase of meiosis II. Following the completion of meiosis I, it is critical to reverse both of these modifications to the cell cycle machinery to enable an equational division during meiosis II. The vertebrate protein Meikin regulates meiosis I co-orientation and centromere cohesion. However, the molecular basis for Meikin's contribution to meiosis remains poorly understood. In particular, the mechanism by which Meikin is inactivated during exit from meiosis I to reverse its activities is unclear. Here, we identify a conserved Separase target site in Meikin that directs Meikin cleavage at anaphase I onset. To determine the consequences of this cleavage event, we analyzed the sequence requirements for Meikin's interaction with kinetochores and regulatory components. We identify motifs in Meikin that are necessary and sufficient for kinetochore localization through an interaction with the inner kinetochore protein CENP-C and define the basis for its interaction with the cell cycle kinase Polo-like kinase 1 (PLK1). Our findings demonstrate that Separase cleavage separates these key domains abolishing Meikin-based regulation of PLK1 activity. Importantly, we find that Separase-resistant Meikin is retained at kinetochores until meiosis II in mouse oocytes, and oocytes expressing Separase-resistant Meikin display defective chromosome alignment during meiosis II consistent with incorrect sister kinetochore orientation. These data demonstrate that Meikin is a novel meiotic Separase substrate and indicate that Separase activity constrains Meikin localization and activity to meiosis I, thus allowing proper meiosis II chromosome segregation.

P236/B242

A Degron-based Strategy Reveals Processes Dependent on Aurora B Kinase Activity in *C. Elegans*.

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The conserved kinase Aurora B facilitates many important events during cell division. While numerous Aurora B substrates have been identified, some functions of Aurora-family kinases do not require kinase activity. Thus, understanding this key class of cell division regulators requires strategies to distinguish kinase-activity dependent and independent functions. We have developed a new degron-based approach that addresses this need, yielding new insights into how Aurora B facilitates cell division in *C. elegans. In C. elegans*, Aurora B (AIR-2) and other members of the chromosome passenger complex (CPC) are required for chromosome segregation and cytokinesis during both mitosis and oocyte meiosis. Moreover, the CPC is required for the assembly of an essential meiotic protein complex that forms a ring around the center of each chromosome in oocytes. This ring complex (RC) consists of over fifteen proteins, and AIR-2 is thought to serve as a scaffold upon which other RC proteins assemble. Furthermore, SUMOylation of some RC components enables the recruitment of others with SUMO-

interacting motifs (SIMs); since AIR-2 can be SUMOylated in *vitro*, AIR-2 SUMOylation could act as an early step in building this SUMO-SIM network (1). However, whether AIR-2's kinase activity is also important for RC assembly or for any of its other meiotic or mitotic functions is not known. Using our degron-based strategy, we inhibited the kinase activity of AIR-2 in *vivo* and determined that its kinase activity is essential for proper RC assembly, demonstrating that AIR-2 does not solely play a scaffolding role in the complex. In conditions where only kinase-dead AIR-2 was present, other CPC components loaded onto chromosomes but were unrestricted to the ring region, demonstrating that kinase activity is required for proper CPC patterning. Additionally, SUMO and SUMO-dependent RC components were absent from the chromosomes. Instead, we observed SUMO aggregates around the spindle, suggesting a role for AIR-2-dependent phosphorylation in the SUMOylation pathway and in SUMO-dependent RC assembly. Finally, we found that AIR-2's kinase activity is required for the proper assembly of the oocyte spindle, independent of AIR-2's role in RC formation, and for chromosome segregation during both meiosis and mitosis. Thus, our studies have revealed that AIR-2 kinase activity is required for multiple essential events in *C. elegans*, providing new insights into how Aurora B regulates cell division. In the future, this strategy can be applied to additional proteins, setting the stage for analogous studies of other essential kinases required for cell division. 1) Pelisch et al. (2017), *Molecular Cell*.

P237/B243

Regulation of Aurora B Kinase Activity by Chromosome Passenger Complex Coacervates.

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It is unclear how cells initiate kinase signaling at the correct time and place. The inner centromere is a region of every mitotic chromosome that imparts unique functions, including mitotic signaling, cohesion, and the building of chromatin that can withstand the pulling forces of kinetochore microtubules. The chromosome passenger complex (CPC) is abundantly localized to the inner centromere in mitosis where it is critical for chromosome segregation and cytokinesis. The CPC is a hetero-tetrameric complex composed of INCENP, Survivin, and Borealin as well as the autoactivating kinase, Aurora B. We have recently found that the Borealin subunit has liquid demixing properties and drives the formation of a non-membranous organelle at inner centromeres. We suggest that the initiation of kinase signaling requires concentration of kinases inside non-membranous organelles, which stimulates kinase activity. In order to test this hypothesis, we are investigating how Aurora B kinase activity is affected by Borealin-mediated phase separation in *vitro*. We have initially focused on a system that physically separates the phase-separating and kinase subcomponents of the CPC. The N-terminus of INCENP, Survivin, and Borealin (ISB) phase-separate at concentrations below those found at inner centromeres. These coacervates recruit a complex of Aurora B and the C-terminus of INCENP (AI). Our system utilizes an Aurora B-specific PhosphoSens® substrate that is found both inside and outside coacervates at relatively similar concentrations and can be used to follow kinase activity, in real time. We have found that ISB coacervates promote the kinase activity of both AI and CPC when compared to similar concentrations of an ISB mutant that is deficient at coacervate formation. We think this is an allosteric activation since we are starting with a fully active kinase. We will use this system to follow kinase autoactivation and stimulation by cofactors including RNA, chromatin, and microtubules.

P238/B244

Function of Microtubule Binding by the Chromosomal Passenger Complex in Chromosome Biorientation.

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One of the key steps during cell division is the attachment of sister chromatids to opposite spindle poles, a process called chromosome biorientation. Sister chromatids are attached to the microtubule-based spindle via the kinetochore, which is a multiprotein network forming at the centromeric region of chromosomes that establishes direct microtubule attachments. Chromosome biorientation is a stochastic process. Initially many erroneous kinetochore microtubule attachments are formed where both sister chromatids are attached to microtubules emanating from the same spindle pole (syntelic attachment). If not efficiently detected and corrected, such syntelic arrangements lead to chromosome missegregation resulting in an unequal distribution of chromosomes. An increased rate of chromosome missegregation (chromsomal instability or CIN) cause aneuploidy, which is detrimental for the cell. In order to maintain a stable genome, the conserved chromosomal passenger complex (CPC) detects and corrects such syntelic attachments during prometaphase. If the CPC encounters a syntelic arrangement, the kinase subunit Aurora B (Ipl1 in yeast) phosphorylates key kinetochore targets leading to detachment of the microtubule so that a new, correct attachment may be formed. Although the CPC phosphorylates the kinetochore in prometaphase, the CPC is mainly localized at the inner centromere until anaphase onset, at which point it relocalizes to the mitotic spindle. Previously, we and others have shown that the centromere localization is dispensable for CPC function in yeast and chicken cells. Interestingly the microtubule localization is essential for chromosome biorientation. In this work we aim to determine the function of CPC microtubule localization in chromosome biorientation. To do so, we focus on the CPC scaffold protein INCENP (Sli15 in yeast), which is responsible for CPC localization via its centromere targeting domain (CEN-box) as well as its microtubule binding domain (MTB). Engineering different MTB mutants, we found that the MTB is necessary for CPC function. Surprisingly, microtubule binding is not sufficient, suggesting an additional function of the MTB.

P239/B245

Identification of Suppressors of Chromosomal Passenger Complex Mutants in Budding Yeast.

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The chromosomal passenger complex (CPC) is responsible for destabilizing incorrect kinetochore-microtubule attachments. Without a functional CPC, microtubule-kinetochore attachments become over stabilized and high levels of missegregation (also known as chromosomal instability or CIN) are induced. CIN leads to aneuploidy and induces loss of proliferation. Even though CIN inhibits growth, many cancers exhibit high levels of CIN and aneuploidy. How cancer becomes tolerant to CIN, despite it being detrimental to normal cells, is still largely unknown. To probe the mechanisms that assist adaptation to CIN, multiple *S. cerevisiae* strains were created that lack the CPC subunit *BIR1,* thus inducing persistent CIN. The bir1Δ strains were then given time to adapt to persistent CIN in liquid culture, and Next-Generation Sequencing was used to identify a number of suppressor mutants of the bir1Δ phenotype. Suppression of the bir1Δ phenotype was confirmed by introducing the mutations prior to *BIR1* deletion. Four categories of suppressor mutants were identified. The first group of bir1Δ suppressor mutants are in the outer kinetochore proteins, including multiple members of the Dam1 complex. Results suggest that this first group of suppressors act at the most downstream part of the kinetochore-microtubule

error correction pathway. The second group of suppressors were in the Spindle Assembly Checkpoint kinase. Like mutants of the CPC, loss of *MPS1* function leads to syntelic-attachments and chromosome missegregation. It is therefore quite surprising that compromising *MPS1* activity would suppress, rather than enhance, the CPC mutant phenotype. The third group of suppressors are in subunits of the SCF ubiquitin ligase complex, which controls entry into S-phase and mitosis. There is currently no known function for the SCF complex in regulating kinetochore microtubule attachments. The last group of suppressor mutants were found in the CPC itself. Interestingly, all these suppressor mutants are in essential genes, indicating that the alleles are either hypomorphic or gain-of-function. These suppressors may help adaptation to CIN through destabilization of the over stabilized kinetochore-microtubule attachments caused by disruption of the CPC. This model is currently being tested by microscopy.

P240/B246

Structural Characterisation of KKT4: an Unconventional Kinetochore Protein.

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A key structure involved in the process of chromosome segregation in eukaryotes is the kinetochore, a proteinaceous complex that mediates the interaction between centromeric DNA and spindle microtubules. Although it was widely assumed that kinetochore components are conserved across all eukaryotes, a unique set of kinetochore proteins was discovered in an evolutionarily-divergent group of organisms called kinetoplastids. No significant similarity to conventional kinetochore proteins is found in these kinetoplastid kinetochore proteins (KKT1-20 and KKIP1-7), and very little is known about their function and structure. Recently we reported KKT4 as the first kinetochore protein in *Trypanosoma brucei* that has microtubule-binding activities. Here we show that its microtubule-binding domain consists of an N-terminal coiled-coil fold and a disordered C-terminal tail that enhances microtubule-binding activities. Interestingly, we discovered that in addition to microtubule-binding activities KKT4 also has DNA-binding activities. Therefore, KKT4 is a unique kinetochore protein in eukaryotes, binding both DNA and microtubules. By analysing KKT4 protein, we aim to understand how the unconventional kinetoplastid kinetochores interact with microtubules to facilitate faithful chromosome segregation.

P241/B247

Crispr-cas9-based Tiling Screens Reveal Protein Domain Structure and Novel Functional Regions in Human Mitotic Factors.

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A critical knowledge gap for the human genome has arisen from our inability to resolve important functional domains and motifs within protein coding genes at the large scale. Historically, large scale annotation of protein domains and motifs relied on homology based-inference by searching against the current 5494 conserved protein family (Pfam) domains documented in the human genome (e.g., methyltransferase-like domain). This approach is ineffective for the ~45% of the proteome that is devoid of Pfam domains, and still requires validation for the remaining genes. Closing this knowledge gap is critical for both basic and disease-focused biomedical research, where years, if not decades, can be spent dissecting gene functions. Here we leverage the mutagenic properties of CRISPR-Cas9 by saturating sgRNAs across the coding sequence of a gene to identify essential domains and motifs. A

panel of diploid and aneuploid cells suggest critical, phenotypically constrained regions do not tolerate in-frame CRISPR-Cas9 indels. As a result, each gene produces a unique mutational signature, with constrained regions scoring as phenotypic "peaks". As a test case, we performed tiling mutagenesis to resolve the domain structure for 48 well characterized kinetochore-associated genes and identified approximately 160 functional regions, of which nearly 1/4 have not been previously described. Novel regions were found in a diverse set of mitotic factors including: SKA3, Rod, Spindly, Shugoshin, Mad1, and chTOG and preliminary evidence suggests many of them contribute to protein-protein interactions responsible for sub-cellular localization. This powerful genetic approach allows rapid and inexpensive dissection of essential protein activities expanding our understanding of genic structure for application to both basic science and disease-focused questions.

P242/B248

Human Alpha1-antitrypsin Intracellular Form Enriches the Nucleus in Interphase and Spindle/kinetochores in Mitotic Cells.

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Alpha1-antitrypsin (AAT) is a potential biomarker in many tumors. The protein level tends to rise in tumor patients. Not only the full-length AAT is associated with cancer progression, but also AAT-derived C-terminal peptides have been found in cancer patients biological fluids [Cercek L & Cercek B, 1992; Chang WC et al., 2008; Zhou J et al., 2010]. One of the established roles of these peptides is the suppression of cytotoxic immune cells in favor of tumor progression [Cercek L & Cercek B, 1993; Kataoka H et al., 1999]. Sporadic findings also showed that AAT could be retained in the cytoplasm of the transformed cells [Normandin K et al., 2010; Shapira MG et al., 2014]. We hypothesized that cancer cells produce intracellular AAT forms (full-length or the C-terminal peptides) along with the secretory counterpart since the dual localization of secretory proteins is an established phenomenon. Here we investigated the DU145 cell line by immunofluorescence using antibodies against the full-length AAT molecule and its C-terminus. Both antibodies reveal protein accumulation in the nucleus, predominantly in euchromatin and the interchromosomal space, but not in the nucleoli and heterochromatin. When cells progress into mitosis, the fraction of AAT detected exclusively by anti-C-terminal antibodies arises as a *bona fide* component of the outer kinetochore, that can be visualized after cell permeabilization. The full-length AAT anchors to the mitotic spindle and spindle poles. We have found that this AAT intracellular distribution is RNA-dependent, so the non-secretory AAT is the RNA- or RNP-binding protein. Since the AAT C-terminal domain has a strongly basic pI in contrast to the rest AAT molecule and contains the predicted RNA-binding motif, we assume that this domain is crucial for exerting AAT functions inside the cell. Indeed, after cell permeabilization, we demonstrated that TAMRA-labeled C36, the synthetic AAT C-terminal peptide, enriches the nucleus, especially the nucleoli, in the interphase cells, and mitotic spindle/spindle poles in the dividing cells, - in contrast to the Cy3-labeled AAT from blood plasma. Although the precise role of intracellular AAT in the cell cycle is to be elucidated, we believe that our study reveals the new facet of AAT functions. The study was supported by RFBR project

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P243/B249

Modeling Chromosome Dynamics and Mitotic Cell Fate Decision in 3d Using Organoids and Mitotic an tagonists.

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Error-free cell division depends on the accurate assembly of the spindle midzone from dynamic spindle microtubules to ensure chromatid segregation during metaphase-anaphase transition. However, the mechanism underlying the key transition from the mitotic spindle to central spindle before anaphase onset remains elusive. Given the prevalence of chromosome instability phenotype in gastric tumorigenesis, we developed a strategy to model context-dependent cell division using a combination of light sheet microscope and 3D gastric organoids. Light sheet microscopic image analyses of 3D organoids showed that BubR1 and CENP-E inhibited cells undergoing aberrant metaphase-anaphase transition and exhibiting chromosome segregation errors during mitosis. Using a selective BubR1 kinase antagonist Bubristatin, we found that inhibition of CENP-E phosphorylation is shown to prevent proper microtubule capture at kinetochores and, surprisingly, proper assembly of the central spindle at mitotic exit. These findings reveal a previously uncharacterized role of BubR1-elicited phosphorylation of CENP-E in temporal control of central spindle assembly.

P244/B250

Meiotic Drive of Non-centromeric Locus R2d2 in Mouse Oocytes.

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Mendel's Law of Segregation states that each allele has an equal chance to transmit to the gametes. However, this law can be violated by selfish genetic elements, which manipulate the production of gametes to increase their own rate of transmission. This genetic cheating in meiosis, meiotic drive, is typically associated with fitness cost to the host and has significant impacts on genetics, evolution, and reproduction. In female meiosis, selfish elements bias their transmission by preferentially segregating to the egg. We focus on selfish R2d2, a non-centromeric locus on mouse chromosome 2, which shows over 90% transmission ratio distortion with mild embryonic lethality. The underlying cell biological basis for both biased segregation and the embryonic lethality is unknown. Here, we developed a strategy to track the R2d2 locus by live imaging in mouse oocytes and found that the chromosome with R2d2 locus lags in anaphase. An aphase lagging could induce biased segregation in oocytes, because the majority of the cytoplasm remains in the egg after polar body extrusion. An aphase lagging would also cause aneuploidy in the egg, leading to embryonic lethality. Therefore, this simple model has a potential to explain the mechanisms underlying both meiotic drive and the associated fitness cost.

P245/B251

Meiosis-Specific Aurora Kinase B Function in Regulating Protein Levels in Mouse Oocytes.

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In mitosis, Aurora kinase B is an essential regulator of the chromosomal passenger complex required for successful chromosome segregation. In mouse oocytes, which are completing meiosis I, Aurora kinase C appears to take over this CPC role and carry out AURKB mitotic-like functions. We previously demonstrated AURKB is required to negatively regulate AURKC, and this negative regulation is required to prevent aneuploidy. How AURKB negatively regulates AURKC is unknown. Oocyte meiosis and early embryo development occur in absence of transcription and depend on the translational activation of accumulated and stored maternal mRNAs. Because transcription is silent during meiosis, we hypothesized that AURKB affects either protein translation and/or protein stability to negatively regulate AURKC. Using a Click chemistry-based translation assay, we found a significant increase in total translation in *Aurkb*knockout (KO) oocytes compared to WT. These findings suggest that AURKB negatively regulates translation during mouse oocyte meiosis.Translation in mouse oocytes is regulated, in part, through the presence of cytoplasmic polyadenylation elements (CPE) located within the 3'UTRs of stored maternal RNAs. The cytoplasmic polyadenylation element binding protein (CPEB1) is responsible for CPE-mediated translation. CPEB1 is then phosphorylated and is mainly turned-over after completion of meiosis I. This phosphorylation is also required for CPEB1 function. We found that CPEB1 is less stable in *Aurkb*KO oocytes compared to WT. In an attempt to identify differentially translated messages that could be driving the aneuploidy phenotype, we isolated polysome-bound RNAs from WT and *Aurkb*KO oocytes. We found an enrichment of RNAs with 2 or more CPEs in their 3'UTR in*Aurkb*KO oocytes. This enrichment suggests that AURKB negatively regulates messages that are highly translated in a CPE-dependent manner. Interestingly, we also found a decrease in ribosome-bound RNAs of proteins with function related to ubiquitylation/ protein degradation pathways, suggesting alterations in protein stability.In conclusion, this study suggests that AURKB can regulate translation and/or protein degradation during oocyte meiotic maturation, events critical to generating euploid eggs.

P246/B252

Retinoblastoma Protein Absence in Human Cells Induces Genomic Instability and Affects Kinetochore Structure.

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The Retinoblastoma protein 'pRB' is a tumor suppressor, encoded by the RB1 gene. pRB repress genes involved in DNA replication and blocks the G1/S transition. A large percentage of human cancers bear mutations in the pRB pathway, resulting in uncontrolled cell proliferation. In addition to this well-characterized role, RB loss has been also implicated in genomic instability, another cancer hallmark, as it leads to gain or loss of chromosomes in mitosis. The mechanisms underlying this mitotic phenotype are, however, not well understood. Our goal is to better understand role the role of pRB in genomic instability. Here we achieved an 80% reduction in pRB expression by RNAi in RPE1 cells, a non-transformed cell line derived from human Retinal Pigmented Epithelium. pRB depletion increased the duration of mitotic timing and increased the incidence of lagging chromosomes after a monastrol-release, highlighting its contribution to genomic instability. Lagging chromosomes can arise from

deregulated kinetochore-microtubule attachments, defective kinetochore structure or incomplete DNA condensation. Using live-cell imaging-based assays we find that microtubules-kinetochore attachments and DNA condensation were not affected by pRB depletion. In contrast, acute pRB depletion lowered the levels of CENP-A at centromeres, the centromeric histone required for the assembly of kinetochores. Our experiments further suggest that this reduction in CENP-A levels, does not affect kinetochore-attachments directly, but correlates with a reduced ability to correct erroneous kinetochore-microtubule attachments. Interestingly it was recently was reported that human cancer lacking pRB are more sensitive to inhibitors against the Aurora B kinase, a key component of the machinery correcting erroneous kinetochore-microtubule attachments. Our future aim is to test whether this increased sensitivity is due to lower CENP-A levels.

P247/B253

Evidence That CENP-B and CENP-C Suppress Biased Segregation of Selfish Centromere DNA in Female Meiosis.

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Centromere evolution is paradoxical in that both centromere DNA and proteins are rapidly evolving despite the conserved requirement of centromeres for faithful chromosome segregation. The centromere drive hypothesis proposes that maternal and paternal centromeres compete for the egg during female meiosis, and selfish centromere non-coding DNA is evolving to achieve preferential inheritance over the homologous counterpart. Under this hypothesis, centromere proteins recurrently evolve to suppress the fitness cost imposed by centromere DNA cheating. To study centromere drive, we have established a hybrid mouse model (a cross between *Mus musculus domesticus* strains) where centromeres from one strain achieve preferential inheritance over centromeres from the other strain. We hypothesized that selfish centromere DNA recruits more CENP-B, the only known centromere-specific DNA binding protein, to achieve preferential inheritance. In contrast to this initial expectation, CENP-B knock-out and over-expression in our hybrid mice suggested that CENP-B suppresses centromere drive. In order to identify other candidate suppressors of drive, we conducted a molecular evolution analysis that detects signatures of rapid evolution. Limited availability of mouse genomes resulted in low-quality data, motivating us to sequence other Murinae species. CENP-C scored highly in the analysis, and over-expression of rat CENP-C in our hybrid mouse model for drive prevented preferential segregation of selfish mouse DNA in meiosis I. This result suggests that centromere DNA directly recruits CENP-C, and CENP-C evolved to “escape” from centromere DNA. Our combined approach of evolutionary analysis and cell biological experiments support the idea that both CENP-B and CENP-C suppress selfish centromere DNA drive.

P248/B254

Ndc80 Complex Is Essential for the Initial Kinetochore-microtubule Capture during Early Mitosis.

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The capture of sister kinetochores by the spindle microtubules (MTs) is essential to form proper kinetochore-microtubule (kMT) attachments that drive chromosome alignment and error-free chromosome segregation. It was reported that kinetochores are initially captured in early mitosis by dynamically unstable MTs (Mitchison and Kirschner, 1984) which are then stabilized to promote bipolar

spindle formation by a 'search-and-capture' mechanism (Kirschner and Mitchison, 1986). During early mitosis, outer surface of unattached kinetochores transiently expand outward to form a fibrous corona, and associate laterally with the sides of the MTs (Barisic et al., 2014; Kapoor et al., 2006; Magidson et al., 2011; McEwen et al., 1998; Rattner and Bazett-Jones, 1989; Ris and Witt, 1981; Tanaka et al., 2005). In humans, MT-based minus-end-directed motor dynein has been reported to transport laterally-attached chromosomes towards the spindle pole (Li et al., 2007; Rieder and Alexander, 1990; Vorozhko et al., 2008; Yang et al., 2007). The Hec1 subunit of the kinetochore-bound Ndc80 complex has direct MT-binding activity and has been demonstrated to play a central role in stabilizing kMT attachments during mitotic metaphase (Cheeseman and Desai, 2008; Miller et al., 2008; Santaguida and Musacchio, 2009; Wei et al., 2007). However, the precise mechanism of how the different kinetochore MT-binding factors contribute to initial kinetochore capture by MTs during early mitosis is still unknown. By combining genome editing and RNAi approaches, we show that mitotic cells lacking the Ndc80 complex exhibit severe defects in capture of kinetochores by MTs during early mitosis. Further, the kinetochore capture mediated by Ndc80 depends on Aurora B kinase-mediated phosphorylation of its N-terminal unstructured tail domain and the normal functionality of the calponin homology (CH) domain as we find that the respective mutants of Ndc80 are unable to rescue the defects in the capturing of kinetochore by MTs. These findings reveal a previously unappreciated and essential role of Ndc80 complex in initial kinetochore capture by MTs during early mitosis, which in turn is required for proper chromosome congression, alignment and segregation.

Chromosome Organization 1

P249/B255

Cargo Motive Force Is Decoupled from ParA Gradient Maintenance in the F Plasmid ParABS Partition System.

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ParABS DNA partition systems ensure faithful segregation of many bacterial chromosomes and low-copy number plasmids. To gain greater insight into the mechanism of these systems, we characterized F-plasmid $ParA_F$—$ParB_F$ protein complexes bound to DNA in the presence of ATPγS using TIRF microscopy. The proteins formed a complex with a 1:1 ratio in the absence of $parS_F$, a centromere-like DNA sequence to which $ParB_F$ binds, but the $ParB_F$:$ParA_F$ ratio doubled in the presence of $parS_F$. This change in ratio did not occur with a $ParB_F$ bearing a mutation in the conserved Box II region, despite $parS_F$-binding being unaffected. The activation efficiency of the DNA-bound $ParA_F$-ATPase by $ParB_F$ was also enhanced by $parS_F$ in a Box II-dependent manner. A monomeric protein carrying only the $ParA_F$-interaction domain of $ParB_F$ activated $ParA_F$ ATPase cooperatively, and formed a 1:1 complex with $ParA_F$. Based on these observations we propose that local $ParA_F$—$ParB_F$ association-dissociation dynamics are partially uncoupled from the slow ATPase step by an activation energy barrier involving a conformational change within the $ParA_F$—$ParB_F$ complex. This would result in decoupling of the generation of the plasmid cargo motive-force via $ParA_F$—$ParB_F$ interactions from the ATPase step that maintains the $ParA_F$-ATP concentration gradient essential for directed motion.

P250/B256

Chromosome Detachment from the Nuclear Envelope Is Required for Genome Stability in Closed Mitosis.

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Mitosis in metazoans involves detachment of chromosomes from the nuclear envelope (NE) and NE breakdown, whereas yeast maintains the nuclear structure throughout mitosis. It remains unknown how chromosome attachment to the NE might affect chromosome movement in yeast. By using a rapamycin-induced dimerization system to tether specific locus of the chromosome to the NE, I found that the tethering delays the separation and causes missegregation of the region distal to the tethered site. The phenotypes are exacerbated by mutations in kinetochore components and Aurora B kinase Ipl1. The chromosome region proximal to the centromere is less affected by the tether, but it exhibits excessive oscillation before segregation. Furthermore, the tether impacts full extension of the mitotic spindle, causing abrupt shrinkage or bending of the spindle in shortened anaphase. The study supports that detachment of chromosomes from the NE is required for faithful chromosome segregation in yeast and that segregation of tethered chromosomes is dependent on fully functional mitotic apparatus.
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P251/B257

Increased Chromosome Speeds in the Absence of Microtubules In*Mesostoma Ehrenbergii* Spermatocytes May Be Due to Actin and Myosin in the Spindle.

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We study chromosome movement in *Mesostoma ehrenbergii* primary spermatocytes to understand what produces the forces for movement. In this cell there are three bivalents which extend across the center of the cell, and four univalents of two different types, 2 metacentrics and 2 acrocentrics (Ferraro-Gideon et al. 2014; Fuge 1987,1989). In previous work, we described experiments in which chromosome kinetochores moved rapidly towards one single pole in the absence of microtubules (Fegaras and Forer 2018 a and b). After 10µM nocodazole (NOC) depolymerized prometaphase spindle microtubules, all chromosomes detached from one pole and rapidly moved to the other pole at speeds up to 200 µm/min. Since chromosomes stretch out in NOC before detaching, the rapid chromosome movements in the absence of microtubules might conceivably be due to recoil from the tension. We argued that this movement is not due to recoil because chromosomes do not move in a straight line between the two kinetochores (Fegaras and Forer 2018 a), but to test this further, we cut 1, 2, or all 3 bivalents into 2 pieces using a femtosecond laser, both before and during addition of NOC. After treatment with NOC, the kinetochores of the severed bivalents still moved to one pole, at speeds similar to non-severed chromosomes. Since movement is not due to either microtubules or tension, we suggest it may be due to actin and myosin in the spindle, as found in other cells (Forer et al., 2015; Mogessie and Schuh 2017). Perturbation of myosin and actin alters bivalent oscillation speeds in *Mesostoma* (Silverio 2017), and our data implicate actin and myosin in the rapid NOC-induced chromosome movement: after treatment of cells with NOC, inhibitors decreased chromosome speed and increased time to detachment, whereas enhancers increased chromosome speed and decreased time to detachment. In future experiments, we will test this conclusion by staining for myosin, phosphorylated myosin, and actin after the addition of NOC, to see if they are in contact with the moving chromosomes. We will also study these proteins in

not-treated cells to determine if they are asymmetrically positioned within the cell or oriented towards a particular pole.

P252/B258

Chromosome-specific Differences during Meiosis.

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Chromosomal aneuploidy in oocytes is one of the leading causes of infertility and miscarriages in women. It has been well known for many years that chromosome missegregation during meiosis is responsible for producing aneuploidy, and that the rate of missegregation can drastically increase when recombination patterns are altered. Interestingly, aneuploidy rates have been shown to vary between chromosomes, indicating that each chromosome may have unique underlying biology that affects its behavior during various meiotic processes. It is unclear, however, what roles important meiotic complexes may play in regulating this chromosome-specific behavior. The synaptonemal complex (SC) is a conserved meiotic structure that regulates the repair of double-strand breaks (DSBs) into crossovers or gene conversions. Recently, we used CRISPR/Cas9 to create a set of targeted SC mutants in *Drosophila* that exhibit chromosome-specific recombination defects reminiscent of the altered recombination patterns seen in human trisomy. These mutants contain varying in-frame deletions within the same *Drosophila* SC protein, each of which resulted in fragmentation of the SC at three different times: early pachytene, early-mid pachytene, and mid pachytene. With this temporal resolution, we investigated when full-length SC is required for meiotic processes such as clustering of centromeres, pairing, and recombination on a chromosome-specific level. We found that the *X* chromosome displays different recombination phenotypes than the autosomes in response to disruptions in SC structure, suggesting crossover distribution is potentially regulated differently on the *X* chromosome versus the autosomes. We are currently working to uncover the mechanisms that underlie these chromosome-specific recombination defects using next-generation sequencing and super-resolution microscopy. These unique mutants allow us to investigate the role of the SC in regulating chromosome-specific meiotic processes.

P253/B259

The DNA Replication Factor PCNA Provides Directed Regulation of Cohesin Function.

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Genomes undergo structural changes in response to transcriptional activation, DNA repair, chromosome condensation, and sister chromatin cohesion. The cohesin complex supports the formation of each of these various DNA architectures, though the mechanism through which cohesin functions are directed to these different chromatin states remains unknown. Prior findings support a role for DNA replication factors in regulating Eco1/Ctf7 (herein Eco1) function to activate cohesin. Early studies revealed that PCNA promotes Eco1 function such that elevated levels of PCNA rescue *eco1* mutant cell growth defects. More recently, PCNA was found to bind and become acetylated by Eco1. Here, we test the extent to which PCNA overexpression rescues phenotypes (cohesion defects, condensation defects, or both) exhibited by *eco1* mutant cells. Intriguingly, the cohesion defect, but not condensation defect, was rescued in *eco1* mutant cells, suggesting that PCNA promotes Eco1-dependent cohesion in a context-specific fashion at the DNA replication fork. Thus, the context of DNA replication is critical to promote

specific roles for Eco1 and cohesin in sister chromatin cohesion. Interestingly, though overexpression of PCNA rescues *eco1* mutant growth defects, excess PCNA exacerbates the growth of cells that harbor a mutation in the cohesin subunit *mcd1*/*scc1* (herein *mcd1*). The molecular basis of this negative genetic interaction remains unknown. One possibility is that elevated levels of chromatin-bound PCNA adversely impact cohesin loading and acetylation. Our results, however, reveal that excess PCNA does not adversely impact cohesin chromatin binding nor cohesin acetylation. Intriguingly, however, elevated levels of wildtype PCNA increases *mcd1* mutant cell sensitivity to DNA damage. These results reveal that PCNA promotes Eco1-dependent cohesion but increases cohesin mutant sensitivity to DNA damage agents. In combination, our results suggest that the context of DNA replication is critical to promote specific roles for cohesin in sister chromatin cohesion but that changes in DNA replication factors can have adverse effects on a subset of cohesin function.

P254/B260

Dna Damage-induced Transcription Reactivates Eco1 to Establish Cohesion.

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The Eco1 acetyltransferase establishes sister chromatid cohesion during DNA replication before its degradation during late S phase that continues throughout M phase. In response to DNA damage in M phase, however, Eco1 is required to establish Damage-Induced Cohesion (DIC) which is critical for efficient DNA repair. To date, little evidence exists for how Eco1 is reactivated during M phase to establish cohesion during the DNA damage response. Popular models include that the degradation machinery that targets Eco1 is inhibited or that changes in post-translational modifications make Eco1 refractile to the degradation pathway. Surprisingly, our results reveal that the degradation machinery is fully competent in response to DNA damage during M phase. Thus, the modifications that target Eco1 for degradation at the end of S phase and throughout M phase persist despite DNA damage. Instead, our results document that DNA damage during M phase induces new transcription of *ECO1* and at a rate that exceeds the rate of Eco1 turnover, providing for Eco1 protein accumulation and DIC-based DNA repair. Eco1 reactivation during M phase is complex and dose-dependent, however, in that high levels of DNA damage fail to reactivate Eco1. Collectively, these findings document a novel regulatory pathway through which Eco1 reactivation occurs during M phase and in response to DNA damage, but that this DNA damage response pathway is complex and dose-dependent. This study highlights the complex nature through which mutations in cohesin or Eco1/ESCO promote developmental abnormalities and contribute to various diseases including cancer.

P255/B261

Self-dna Sensing by Cgas and Its Effect on Cell Fate during Mitotic Arrest.

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cGAS is a major pattern-recognition receptor detecting cytoplasmic DNA for the innate immune system. Upon DNA binding, cGAS becomes an enzyme, synthesizing a cyclic GMP-AMP dinucleotide second messenger (cGAMP), which promotes inflammation via activation of IRF3- and NF-kB-dependent transcription. The primary substrate for cGAS is pathogen-derived DNA but the mechanisms by which cGAS distinguishes between self-DNA and non-self DNA are poorly understood. Furthermore, cGAS is also thought to respond to a subset of aberrant self-DNA structures such as micronuclei. We have shown

that the organization of eukaryotic nuclear DNA into chromatin may serve as a signal for self-DNA, inhibiting activation of cGAS. The basic unit of chromatin, the nucleosome, binds cGAS with higher affinity than does naked DNA, but nucleosomes competitively inhibit DNA-dependent cGAS activation. Thus, the cGAS pathway is not effectively activated during normal mitosis, when the nuclear envelope disassembles, exposing all chromosomal DNA to the cytoplasmic cGAS. However, during mitotic arrest, low level cGAS-dependent IRF3 phosphorylation slowly accumulates, promoting cell death rather than inflammation. Accordingly, expression of cGAS in cancer cells makes mouse xenograft tumors responsive to the anti-mitotic agent Taxol. TCGA datasets for non-small cell lung cancer patients also suggest an effect of cGAS expression on the taxane response. To determine the mechanism by which nucleosomes inhibit cGAS, we have investigated the biochemistry of the interaction between cGAS and nucleosomes. DNA-binding mutants of cGAS only mildly affect affinity for nucleosomes, suggesting an interaction between histones and cGAS. Indeed, cGAS directly associates with the histone H2A-H2B dimer in a manner dependent on the acidic patch of H2A-H2B, a hotspot for nucleosome interactors.

P256/B262

Gy Controls Meiotic Progression by a Phase Separation Mechanism during Spermatogenesis.

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Meiosis is germ cell-specific cell division that generates haploid gametes in sexually reproducing organisms. During meiosis, environmental stresses can damage meiotic cells and lead to clinical infertility. It has long been observed that in cryptorchidism patients, undescended testes do not provide suitable temperature for spermatogenesis, consequently, meiotic spermatocytes are decreased. However, the molecular mechanism underlying this stress sensitive phenomenon is largely unknown. Here we report that liquid-liquid phase separation of a novel RNA binding protein GY, ensures both meiotic progression and cell fitness under stress conditions. Stress granules were constantly observed in the testes of cryptorchid patients. We found GY to be essential for heat stress-induced granule formation. We provide evidence that GY ensures the meiotic progression through two mechanisms: stabilization of target mRNAs to control translation of meiotic molecules; enhancement of cellular fitness of meiotic cells in stress conditions through an inherent phase separation mechanism. We propose that reversible phase separation of GY ensures meiotic synapsis in spermatocytes under stress conditions.

P257/B263

***Spindlin1* Regulates BUB3 Expression and Affects an aphase Transition during Meiotic Maturation in Porcine Oocytes.**

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Spindlin1 (*SPIN1*), which contains Tudor-like domains, regulates maternal transcripts via interaction with a messenger RNA (mRNA)-binding protein. *SPIN1* is involved in tumorigenesis in somatic cells and is highly expressed in the cancer cell. Nevertheless, the function of *SPIN1* in porcine oocyte maturation remains totally unknown. To explore the function od *SPIN1* in porcine oocyte maturation, knockdown, and overexpression techniques were used. *SPIN1* mRNA was identified in maternal stages ranging from GV to MII. *SPIN1* was localized in the cytoplasm and to chromosomes during meiosis. *SPIN1* knockdown accelerated first polar body extrusion. Oocytes with overexpressed *SPIN1* were arrested at the MI stage. *SPIN1* depletion caused meiotic spindle defects and chromosome instability. The BUB3 signal was

investigated, confirming that *SPIN1* affects the stability of *BUB3* mRNA as well as BUB3 expression. Further, overexpression of *SPIN1* inhibited the degradation and regulation of G2/mitotic-specific cyclin-B1. In summation, *SPIN1* regulates the meiotic cycle by modulating the activation of the spindle assembly checkpoint.

P258/B264

Set Binding to Sgo1 Inhibits Sgo-cohesin Interactions and Promotes Chromosome Segregation.

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At anaphase onset, Sgo1 function of cohesion protection must be disabled to allow timely chromosome segregation, but how this is achieved is not fully understood. Here, we show that SET, a known PP2A inhibitor, directly binds to a domain in Sgo1 in close proximity to the cohesin-binding motif. The Sgo1-cohesin binding can be disrupted by SET in a dose-dependent manner in vitro as well as by SET overexpression in cells, suggesting that SET is also an inhibitor to the Sgo1-cohesin binding. Furthermore, the SET binding-deficient Sgo1 mutant fully supports centromeric cohesion protection but delays chromosome segregation, suggesting that the SET-Sgo1 binding is required for timely chromosome segregation. Moreover, overexpression of SET WT, not the Sgo1 binding-deficient mutant, exacerbates the occurrence of cohesion fatigue in MG132-arrested cells. Conversely, SET depletion delays it. Thus, we propose that a major function of SET during mitosis is to disrupt the Sgo1-cohesin interaction, thereby promoting centromeric cohesion de-protection and timely chromosome segregation at anaphase onset.

P259/B265

Force Measurement and Micromanipulation of Chromosomes in Multiple Systems Using a Newly-constructed Micromanipulator.

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Correct positioning of chromosomes during cell division (mitosis and meiosis) is essential for correct distribution of chromosomes into daughter cells. Failures in positioning and distribution of chromosomes can have catastrophic consequences for the cell and the organism the cell inhabits. Positioning of chromosomes relies on balancing forces experienced by chromosomes through their attachments to the spindle. Understanding what forces are felt by chromosomes attached to the spindle requires that the forces be measured. Mitotic forces have only been measured in a small set of organisms and types of division. We have designed and built a new micromanipulator that has adjustable sensitivity and has ideal features for measuring forces on chromosomes. We used the micromanipulator to move chromosomes in cells in mitosis, meiosis I, and meiosis II. We also used the micromanipulator to stall a chromosome's anaphase movements and have gotten preliminary stall force measurements in a number of systems. Our initial measurements were consistent with those Bruce Nicklas obtained using a similar system (on the order of piconewtons). In the future, we plan to use this micromanipulator to measure forces on different types of chromosomes (e.g. holocentric, monocentric, distance-segregating, oscillating, etc.) in mitosis, meiosis I, and meiosis II.

P260/B266

Actin Destabilization Selectively Causes One Spindle Pole to Rotate and Those Kinetochores to Detach and Migrate to the Opposite Pole in *Mesostoma Ehrenbergii* Spermatocytes.

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Chromosome movement and segregation in meiotic cell division differs from the norm in *Mesostoma ehrenbergii* spermatocytes. The atypical features of their division include a pre-anaphase cleavage furrow and a prolonged prometaphase in which bivalents persistently oscillate between the two poles before entering anaphase. Additionally, these cells are speculated to divide via non-random segregation, a process where sets of chromosomes segregate to a specific pole (Ferraro-Gideon et al 2014). Due to these characteristics, the *Mesostoma* provides a useful model to study chromosome movement and non-random segregation. It is primarily theorized that spindle microtubules drive chromosome movement. However, previous results with *Mesostoma* have shown chromosomes continue to move when microtubules are depolymerized using nocodazole (NOC) (Fegaras and Forer 2018). These results also showed all three bivalents selectively detach and move to one pole, adding evidence to the theory *Mesostoma* spermatocytes divide non-randomly. To determine what proteins may be responsible for chromosome movement in the absence of microtubules and why the movement is one-sided, we targeted actin using the actin-stabilizing drug, Jasplakinolide (Jasp). We targeted actin because it has been found in the meiotic spindle in various other cell types, and is implicated in *Mesostoma* bivalent oscillations (Silverio 2017). We recorded live cells to monitor the effects on chromosome movement. Jasp had no effect on the speed of bivalent oscillations, instead, it caused kinetochores at only one pole to rotate at different angles along the cell membrane. Interestingly, those kinetochores at the rotating pole consistently moved to the opposite pole, in a similar fashion to NOC treated cells. Unlike NOC, the precocious furrow moved towards the pole with more chromosomes, as opposed to fewer chromosomes. We do not yet know why the furrow responds in this manner. In summary, our preliminary Jasp results implicate actin's involvement in chromosome movement, and due to the one-sided movement, our results further suggest these cells divide via non-random segregation. Future experiments will be done using Jasp and NOC in a double drug experiment, to test whether the same pole that changes orientation in Jasp is also the same pole that detaches during NOC treatment. If it is the same pole, this will again implicate actin is involved in chromosome movement and whether there is specificity in pole selection.

P261/B267

An an alysis of Dicentric Chromosome Breakage in Yeast.

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An an alysis of Dicentric Chromosome Breakage in Yeast Centromeres mediate kinetochore and microtubule attachment to sister chromatids and are essential for faithful chromosome segregation. Multiple active centromeres, however, leads to genome instability. While dicentric chromosomes occur naturally, one of the centromeres is usually silenced. In 1939, Barbara McClintock noted that having 2 activated centromeres on a single chromosome was highly unstable and caused double stranded breaks in DNA. Dicentric cells can repair double stranded breaks via a breakage-fusion-bridge cycle that will result in a monocentric derivative, allowing the cell to undergo normal division without further breakage. Because the centromere and surrounding region (pericentromere) contain vital cell machinery, we hypothesize that the pericentromere represents a unique environment within the cell

that uses different repair mechanisms than those used on the chromosome arm. To test this, we created dicentric yeast strains containing the endogenous centromere (Cen3), and a second, conditionally activated centromere (Galcen) that is activated in the absence of galactose. We inserted Galcen at varying distances from Cen3, adjacent to specific nonessential genes flanking the pericentromere, a region which extends approximately 10 kilobases (Kb) beyond the endogenous centromere. Engineered mutations in the Rad52 repair pathway, as well as direct and indirect Galcen polarities were used to better characterize the repair mechanisms used in the pericentromere, such as single strand annealing, ring-rod repair, and dicentric regeneration.

P262/B268

Micronuclei Resulting from Different Insults, Differently Impact Genomic Stability.

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Micronuclei contain chromosomes excluded from the main nucleus and are often used clinically to evaluate genomic instability. Cells that enter cell division with a micronucleus can incur severe, localized DNA rearrangements to the micronucleated chromosome [1,2]. This catastrophic process, termed chromothripsis, has been identified as an early event in tumorigenesis [2-5]. Further, loss of micronuclear envelope integrity can also lead to genomic instability by exposing the chromosome to damage in the cell's cytoplasm [6-7]. We have made the paradoxical discovery that mice with a mutation in the kinesin motor *Kif18a* form micronuclei as a result of chromosome alignment defects but do not develop tumors. Further, *Kif18a* mutant mice display resistance to induced colorectal tumors [8]. We are using a combination of in *vitro* cell culture experiments and transgenic mouse models to understand why micronuclei do not lead to tumorigenesis in *Kif18a* mutant mice. Specifically, we are evaluating the nuclear envelope integrity and division rate of cells containing micronuclei induced by loss of (1) chromosome alignment, (2) merotelic kinetochore microtubule attachments, and (3) DNA double strand breaks, both in the presence and absence of p53 activity. Our data indicate that the probability of micronuclear envelope rupture differs among micronuclei caused by different cellular insults but that the rate of micronucleated cell division is reduced by p53 regardless of the insult. These data suggest that multiple defects may be necessary for a micronucleus to promote genomic instability and increase the likelihood of cellular transformation. 1. Crasta et al., 2012, *Nature*. 2. Zhang et al., 2015, *Nature*. 3. Stephens et al., 2011, *Cell*. 4. Nones et al., 2014, *Nature Communications*. 5. Luijten et al., 2018, *Mutation Research*. 6. Hatch et al., 2013, *Cell*. 7. Liu et al., 2018, *Nature*. 8. Zhu et al., 2013, *Biochemical and Biophysical Research Communications*.

P263/B269

Lysosomal Degradation Ensures Accurate Chromosomal Segregation to Prevent Genomic Instability.

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Lysosomes, as primary degradative organelles, are the end-point of different converging pathways including macroautophagy. To date, lysosome function has mainly focused on interphase cells, while

their role during mitosis remains controversial. Mitosis dictates the faithfultransmission of genetic material among generations, and perturbations of mitotic division lead to chromosomal instability, a hallmark of cancer. Heretofore, correct mitotic progression relies on the orchestrated degradation of mitotic factors, which was mainly attributed to ubiquitin-triggered proteasome-dependent degradation. Here, we show that mitotic transition does not only rely on proteasome-dependent degradation, as impairment of lysosomes increases mitotic timing andleads to mitotic errors, thus promoting chromosomal instability. Furthermore, we identified several putative lysosomal targets in mitotic cells. Among them, WAPL, a cohesin regulatory protein, emerged as a novel p62-interacting protein for targeted lysosomal degradation. Finally, we characterized an atypical nuclear phenotype, the toroidal nucleus, as a novel biomarker for genotoxic screenings. Our results establish lysosome-dependent degradation as an essential event to prevent genomic instability.

6

Cancer Stem Cells

P264/B271

Investigating the Role of Metabolically Linked Cell-Extracellular Matrix Interactions on the Breast Cancer Stem-like Cell Phenotype.

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A subpopulation of breast cancer cells with stem-like characteristics (CSCs) contributes to worse clinical prognosis and is characterized by metabolic plasticity. However, the mechanisms leading to the development of CSCs and the role of metabolism in this process remain to be elucidated. Altered cell-extracellular matrix (ECM) interactions have been shown independently to modulate stemness and metabolic reprogramming, but whether there exists a functional link between cancer cell stemness, metabolism, and adhesion is unclear. Here, we hypothesized that changes in tumor cell metabolism promote a CSC phenotype by altering cell-substrate interactions. To address this hypothesis, we employed an established NANOG-MDA-MB231 reporter cell line (MDA:NANOG) that allows detection of CSCs via increased GFP expression. Seahorse metabolic flux analysis of sorted MDA:NANOG cells revealed that more stem-like, GFP-high cells exhibited increased oxygen consumption and glycolysis relative to more differentiated GFP-null cells. Accordingly, increased glucose concentration increased GFP, while blocking glycolysis with 2-deoxyglucose decreased stem cell marker expression. Image analysis further revealed a negative correlation between GFP expression and cell spreading whereas phosphorylated focal adhesion kinase (pFAK) positively correlated with GFP expression. These results suggested that CSCs exhibit metabolism-dependent differences in mechanotransduction relative to their more differentiated counterparts. Indeed, traction force microscopy elucidated that stem-like MDA:NANOG display a ~2.5-fold decrease in total cellular traction force compared to more differentiated cells. As the glycocalyx is critically involved in cell adhesion, regulates phosphorylation of FAK, is subject to metabolic regulation, and contains hyaluronic acid (HA), a ligand for the CSC marker CD44, we next tested whether the observed stemness-dependent differences in adhesion may be due to altered cell-surface associated HA. Indeed, overexpression of HA synthase 3 in MCF10A not only

increased glycolysis, but also decreased cell spreading, while increasing the activity of the stem cell marker aldehyde dehydrogenase. Correspondingly, higher levels of cell-surface-associated HA correlated with increased GFP expression in MDA:NANOG reporter cells, whereas inhibition of HA synthesis with 4-methylumbelliferone increased cell spreading but decreased GFP expression. Together, our data suggest that the increased glycolytic capacity of breast CSCs increases glycocalyx-HA levels to alter cell adhesive properties, which may help explain why increased HA content in tumors correlates with poor clinical outcome in patients.

P265/B272

Control of the Homeostasis of Endolysosomes by the Paracaspase Malt1 Regulates Glioma Cell Survival.

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Glioblastoma is one of the most lethal forms of adult cancer with a median survival of around 15 months. A potential treatment strategy involves targeting glioblastoma stem-like cells (GSC), which constitute a cell autonomous reservoir of aberrant cells able to initiate, maintain, and repopulate the tumor mass. Here, we report that the expression of the paracaspase mucosa-associated lymphoid tissue l (MALT1), a protease previously linked to antigen receptor-mediated NF-κB activation and B-cell lymphoma survival, inversely correlates with patient probability of survival. The knockdown of *MALT1* largely impaired the expansion of patient-derived stem-like cells in *vitro*, and this could be recapitulated with pharmacological inhibitors, in *vitro* and in *vivo*. Blocking MALT1 protease activity increases the endo-lysosome abundance, impaired autophagic flux, and culminates in lysosomal-mediated death, concomitantly with mTOR inactivation and dispersion from lysosomes. These findings place MALT1 as a new druggable target involved in glioblastoma and unveil ways to modulate the homeostasis of endo-lysosomes.

P266/B273

Characterizing Stem Cell Marker Properties in Non-malignant and Malignant Breast Cancer Cells Under Three-dimensional Conditions.

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Despite the advances in breast cancer detection and treatment, it still remains the second leading cause of cancer-related deaths among women. Therefore, developing more effective therapeutics for the treatment of this deadly disease is imperative. The mechanisms that lead to aberrant cell proliferation, dedifferentiation, and eventually oncogenesis, however, remain largely unknown. Previous studies in the Bissell lab have shown that the cellular microenvironment plays a significant role in mammary gland differentiation and function, particularly, the extracellular matrix (ECM). Similarly, cancer stem cells (CSC) have been shown to contribute to tumorigenesis, disease progression and therapeutic resistance, in a process that is potentially regulated by the ECM. More specifically, CSC markers (CD44+/CD24-/low and CD49f+) initiated tumorigenesis when expressed on the surface of breast cancer cells. However, the ECM exerted effects on these markers were not taken into account since most of the initial studies were performed under 2D conditions. Therefore, our goal was to characterize the expression patterns of these CSC markers in the HMT3522 breast series of cell lines under more physiological 3D conditions. We hypothesized that malignant HMT3522 breast cancer cells, and not their non-malignant

counterparts, will exhibit enhanced stem-like properties similar to those associated with tumor formation in mice. To test this, we first compared CD24, CD44 and CD49f expression across different cancer subtypes using deposited TCGA patient data. Then, we correlated these markers' expression in breast cancer patients to survival. Our results showed increasingly higher alteration frequencies in CD24, CD49f, and CD44 among all cancer subtypes with gene amplification being the most common type of alteration. RNA expression of these markers was also determined in breast cancer cell lines, under 2D and 3D conditions, using microarray and qPCR. These findings were then validated using western blot analysis. Significant differences in CD24, CD44, and CD49f expression were observed in malignant when compared to non-malignant cells and those treated with growth blocking inhibitors (GBIs), but only under 3D conditions. Phase-contrast microscopy and immunofluorescence imaging were then used to determine the phenotypic differences between these cells, under the same conditions. Treating malignant cells, cultured in 3D environments, with GBIs induced the formation of structures that resemble normal mammary glands that exhibited decrease stem cell properties. Together, these findings validate the expression of CSC related markers in our 3D model. With this information, we can now begin to elucidate the mechanisms that regulate CSC growth and therapeutic resistance.

P267/B274

Cancer Stem Cells Model as the Origin of Tumor-associated Macrophages in Tumor Microenvironment.

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Abstract: Aim of the study: Tumor-associated macrophages (TAMs) are the most prominent immune cell in the stromal compartment of the tumor microenvironment of solid tumors. Their presence associates with worse prognosis in several cancers. The origins of macrophages in many cancers is still obscured. The present study displayed histological and immunohistochemical analyses of a malignant tumor model developed from cancer stem cells (CSCs) converted from human induced pluripotent stem cells (hiPSCs) in cancer microenvironment prepared by the conditioned medium (CM) of cancer cell line. Especially we focused on the localization and the origin of TAMs, as the furthermost important immune cells and vital source of iron in tumor microenvironment (TME). To the best of our knowledge this may be the first study to suggest the potential differentiation of CSCs to TAMs. **Material and methods:** CSCs were established by culturing hiPSCs in the CM prepared from human pancreatic carcinoma cell line PK8 cells. The markers of stemness and CSCs were evaluated by RT-qPCR. Then CSCs were transplanted and formed primary tumors in immunocompromised mice. The primary culture was serially transplanted again to obtain the secondary tumors. Tumor tissues were subjected to histological, histochemical, immunohistochemical, immunofluorescence, morphometric and statistical studies. **Results:** hiPSCs were converted into CSCs in the presence of CM from PK8 cells. The primary tumors were teratoma while the secondary tumors displayed histopathological features of malignancy. Cells derived from both tumors maintained the expression of endogenous stemness markers, such as Nanog, Oct3/4, Sox2 and c-myc and pancreatic CSCs markers, CD44 and CD133. Tumor sections showed high expression to Ki67, CK19, PTF1A and Sox2 immunohistochemical positive cells. Simultaneously, high immunoreactivity to both anti-human and anti-mouse CD68 antibodies were detected revealing that the tumor tissue derived from CSCs was enriched by macrophages originated from both human and mouse cells. Histological and histochemical examination of tumor tissue showed hemosiderin iron deposits inside several

macrophages. **Conclusions:** the model of CSCs converted from hiPSCs highlighted the importance of the localization and the origin of TAMs as prognostic markers of malignancy. TAMs and tissue-resident macrophages can be regarded as the main link of iron homeostasis in TME. Our study model provides a useful new tool for TAMs research. Further studies of CSCs and TME will provide novel views of cancer development and the ideas of cancer therapies targeting CSCs and TME. **Keywords:** TAMs, TME, Cancer stem cells, hiPSCs, malignant tumor, CD68, hemosiderin iron.

P268/B275

Investigating the Effect of Cannabidiol on Cancer Cells.

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Martin Olmos Whittier College Investigating the effect of Cannabidiol on Cancer Cells Cannabis, although commonly associated with its use as a psychoactive drug, also has been shown to alleviate pain and disease symptoms. In particular, Cannabidiol (CBD), a constitute of Cannabis, has demonstrated numerous benefits such as acting as an anti-inflammatory, anticonvulsant, and antioxidant. Studies exploring the benefits of CBD in cancer have shown its ability to trigger the induction of ER stress and inhibition of AKT and mTOR signaling in breast cancer cells, and the upregulation of Beclin 1 triggering apoptosis in melanoma. Taken together these studies suggest that CBD's cytotoxic and anti-proliferative properties may be suitable as a treatment for cancer, and our previous studies demonstrated that CBD exposure as low as 5 ug was effective in statistically decreasing viability in four cancer cell lines, two tumor forming cell lines (LNCaP prostate cancer and MCF-7breast cancer), and two non-tumor forming cancers (Jurkat T-cell leukemia and CML myeloma). Therefore, this current study aimed to explored if lower exposures of CBD could also induce death, and also sought to determine the cellular pathway through which the cells were dying. Using viability and biochemical reporter assays, our findings show the CBD was successful in statistically reducing viability at even low concentrations (1 ug, 2 ug, 4 ug, and 6ug) when exposed to CBD for as little as 24 hours. Using a real time apoptosis/necrosis assay, we also found that 5 ug of CBD exposure induced apoptosis in the MCF-7 cells, but mainly necrosis in the others, LNCaP, Jurkat, and CML cells. The results of these assays were also supported through RT-PCR analysis demonstrating an increase in gene expression of BAX, BCL-2 and BNIP3 and RIP3 respectively. Taken together, these findings lend to the increasing evidence of CBD as a potential viable, alternative, and less harmful treatment option against both adherent and non-adherent cancers.

P269/B276

Histone Demethylase Jmjd2d Promotes Stemness of Liver Cancer Stem Cells through Wnt and Notch Signaling Pathway.

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Liver cancer is a highly malignant cancer containing cancer stem cells (CSCs) which contribute to high rate of heterogeneity, metastasis, therapeutic resistance and tumor recurrence resulting in poor outcome and limited therapeutic options. JMJD2D is a Histone Demethylase expressed in some solid tumors, including HCC. However, the molecular mechanisms of JMJD2D in liver CSCs remain unclear. Here, we found that JMJD2D is overexpressed in human liver cancer tissues (52%) and liver cancer stem cells (LCSCs) and indicates poor prognosis. Depletion of JMJD2D markedly inhibited LCSC initiation and progression in *vitro and in vivo*. Furthermore, JMJD2D depletion inhibited Circulating tumor cells (CTCS) and lung metastasis of LCSCs. Mechanistically, JMJD2D promotes Wnt target gene Epcam and Notch

target gene Sox9 expression through interacting with β-catenin/TCF4 and NICD to reduce H3K9me3 levels at the promoter respectively. Finally, the JMJD2D inhibitor 5-c-8HQ can attenuate LCSC stemness in *vitro and in vivo*. Collectively, our findings suggesting that JMJD2D is essential for the LCSCs maintenance of self-renewal and JMJD2D could be a possible therapeutic target against LCSCs.

P270/B277

Lineage Tracing Nucleotide Polymorphisms Via Whole Genome Sequencing in Normal, Premalignant, and Malignant Mammary Populations.

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Understanding the development of the normal mammary gland and the role of genetic variations in transforming mammary epithelium towards malignancy is key to providing insight for breast carcinogenesis. In our experimental model, normal, premalignant, and malignant tissues may be derived from the same original cell. These experiments were conducted using the Czech II mouse model to take advantage of their lack of endogenous mouse mammary tumor virus (MMTV). Thus, it was possible to track somatic clonality through viral DNA insertions in the nuclear cellular DNA. These observations have been confirmed through Southern Blot analysis and recently verified through whole genome sequencing. In the present study, we employ whole genome sequencing analysis in an attempt to determine whether genomic polymorphisms define the differences between the premalignant and "normal" outgrowth clones and the mammary tumors that stochastically develop in these cellular populations. We hypothesize that mammary populations may conserve genomic nucleotide polymorphisms in oncogenic progression via selection of clonal progeny arising from the originally infected cell. Genomic DNA (gDNA) was therefore isolated from normal, premalignant, and malignant clonal popuations to determine by whole genome sequence (WGS) the presence of nucleotide polymorphisms and their frequency during oncogenic progression. Our preliminary results were directed mainly toward genomic inheritance of nucleotide polymorphisms in mammary tumors derived from a single premalignant lesion to uncover possible genetic changes associated with oncogenic progression from premalignancy to tumorigenesis. Genes such as Bfsp1, Pappa, Gm11487, EIF3e, Dock4, Pdzm4, and Spear1 were identified as harboring nucleotide polymorphisms among multiple pairs of tumors and metastases but not detected in the normal lactating CzechII mammary gland or the original hyperplasia. Studies are currently underway to determine the impact and consequences of these polymorphisms in tumor progression. Our findings may provide insight into pathways underlying tumorigenesis and lead to development of improved molecular anti-cancer treatment strategies.

P271/B278

Metabolic Coordination of Stem Cell Fate Drives Tumor Initiation.

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Tissue stem cells are the cell of origin for many malignancies. Metabolites regulate the balance between self-renewal and differentiation, but whether endogenous metabolic pathways predispose stem cells to transformation in *vivo* remains unknown. Here, we tackle this question in epidermal stem cells (EpdSCs), a cell of origin for squamous cell carcinoma (SCC). Using genetics, cell and molecular approaches, metabolic profiling and dietary interventions, we find that *de novo* serine synthesis promotes epidermal differentiation. We show that oncogenic EpdSCs restrict glucose-derived serine synthesis, and in doing

so, limit production of alpha-ketoglutarate that drives H3K27me3 loss and epidermal differentiation. When extracellular serine is limiting, enforced serine synthesis induces differentiation and stunts SCC growth, whereas blocking serine synthesis or antagonizing alpha-ketoglutarate-driven demethylation facilitates malignant progression in *vivo*. Thus, metabolic reprogramming that limits serine synthesis supports oncogenic self-renewal and renders SCC initiation sensitive to dietary serine manipulation. These results demonstrate that a stem cell's intrinsic metabolic program dictates its capacity to initiate a neoplasm.

P272/B279

The Tetraspanin CD82 Regulates $S1PR_1$ Mediated Hematopoietic Stem and Progenitor Cell Mobilization.

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The mobilization of hematopoietic stem and progenitor cell (HSPCs) from the bone marrow into the blood occurs under normal physiological conditions and can be stimulated in the clinic to enable the isolation of HSPCs used for transplantation therapies. HSPC transplants are critical treatments for hematological diseases, and thus methods to improve HSPC isolation are of significant interest. Recent work from our laboratory identified the tetraspanin CD82 as a novel regulator of HSPC mobilization. Using a global CD82 knock out (CD82KO) mouse model, we measured enhanced mobilization of HSPCs within CD82KO mice following treatment with the mobilizing agent AMD3100. To determine whether enhanced mobilization was due to CD82KO in the HSPCs rather than the bone marrow microenvironment, C57Bl6 and CD82KO HSPCs were transplanted into lethally irradiated BoyJ mice. Once the bone marrow transplant was established, mice were treated with AMD3100 and similar to previous results, CD82KO HSPCs displayed enhanced mobilization. These data indicate that CD82KO in the HSPCs specially promote enhanced mobilization. Next, we went on to assess the mechanism of enhanced mobilization. While we saw no change in CXCR4 expression or activity, flow cytometry analysis indicates that CD82KO HSPCs have increased surface expression of sphingosine 1-phosphate receptor 1($S1PR_1$), a G-coupled protein receptor that elicits downstream cellular activities such as migration, proliferation and cytoskeletal rearrangement. To confirm a role for $S1PR_1$ in the enhanced mobilization phenotype of CD82KO HSPCs, we performed mobilization assays with the addition of two $S1PR_1$ antagonists 4-deoxypyridoxine (DOP) and FTY720. Upon both FTY720 and DOP treatment, the number of mobilized CD82KO HSPC is decreased when compared to AMD3100 treatment with no difference in mobilization detected between WT and CD82KO mice. These data suggest that the enhanced mobilization of CD82KO HSPCs is dependent upon $S1PR_1$. Current studies aim to identify the mechanism by which CD82KO HSPCs have increased expression of $S1PR_1$ by analyzing $S1PR_1$ transcription factors and using Imagestream cytometry to assess $S1PR_1$ internalization. To identify possible clinical applications, we used a neutralizing CD82 antibody (anti-CD82) to see if blocking CD82 could illicit enhanced mobilization. While anti-CD82 alone shows no effect on white blood cell (WBC) or HSPC mobilization, mice treated with anti-CD82 in combination with AMD3100 display increased WBC and HSPC cell mobilization when compared to controls. Together, these data provide evidence that CD82 is an important regulator of HSPC mobilization and suggests exploiting the CD82 scaffold as a therapeutic target to enhance stem cell mobilization treatments.

P273/B280

Establishment of Cancer Stem Cell Model Overexpressing *BAMBI* and *PORCN and* Attenuating *SFRP1*.

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Background: We have previously proposed a method to develop cancer stem cells (CSCs) from induced pluripotent stem cells (iPSCs) through culture with conditioned media (CM) derived from cancer cells. This is an efficient way to obtain CSCs which potentially represent similar CSCs to in *vivo* populations and will allow the study of inducing events, survival and disease progression. We hypothesized that this method could be extended to ovarian cancer (OVCA) lines. Methods: We utilized human iPSCs (NipsB2) and CM from three different OVCA lines (A2780, MDAH2774, and SKOV3) and analyzed the resultant CSCs by qPCR and RNAseq. To determine tumorigenicity, we examined the cells' ability to form spheroids in *vitro and* utilized an axillary xenograft model in NOD.*Cg-Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) mice. Results: All of the NipsB2 derived CSCs (NipsB2-CSCs) expressed stem cell (*NANOG* and *ALDH1*) and CSC (*CD44* and *CD133*) markers and developed spheroids in culture and tumors in NSG mice. RNAseq analysis revealed, in all of the derived CSCs, overexpression of BMP activin membrane-bound inhibitor (*BAMBI)*, a pseudoreceptor of TGFβ, and porcupine (*PORCN*), a WNT acylating enzyme, and downregulation of secreted frizzled-related protein 1 (*SFRP1*), a tumor suppressor. Conclusions: Our NipsB2-CSC demonstrate prototypical gene expression changes seen in primary CSCs isolated in *vivo*. Our results suggest that the NipsB2-CSCs are a model which can be studied further for CSC initiation, survival and disease progression.

P274/B281

Correlation between Sox2 Protein Expression and Prognosis of Lung Cancer Patients.

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The pluripotency-associated transcription factor SOX2 (sex determining region Y-box 2) has been known as a master factor regulating stemness, and several reports suggested that SOX2 played a crucial role in the maintenance of lung cancer stemness. However, there have been discrepancies in the correlation between SOX2 protein expression and patient prognosis, especially in non-small cell lung cancer (NSCLC) adenocarcinoma (LUAD). To clearly understand the relationship between SOX2 and NSCLC, we collected a total of 545 NSCLC patient's samples (287 of LUAD, 243 of lung squamous cell carcinoma (LUSC), and 15 of others) and its clinicopathological information, and analyzed the correlation of the expression levels of SOX2 and the prognosis of NSCLC. As a result, we could find that the expression level of SOX2 is not associated with the prognosis after surgery in NSCLC patients. To further demonstrate our results, we modulated SOX2 protein level by using doxycycline-inducible small hairpin RNA (shRNA)-mediated knockdown and CRISPR/Cas9-mediated knockout systems in two LUAD cell lines. Knockdown of SOX2 by shRNA significantly affected LUAD cell proliferation and tumor sphere formation, but this phenotype was not rescued by the restoration of SOX2. Knockout of SOX2 by the CRISPR/Cas9-system did not result in significant differences in cell proliferation, colony forming assay, tumor sphere formation, and chemoresistance. Overall, this study suggested that SOX2 protein expression is not correlated with lung cancer patient prognosis and care should be taken to generalize SOX2 as a stemness maintenance factor in various cancers.

P275/B282

Comparison of Label Free Imaging Approaches and Destructive in *Vitro* Cell Based Assays for the Assessment of Growth and Viability of Primary Patient-derived Organoid Cancer Models.

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One major challenge in cancer biology is the lack of physiologically relevant models that have long term expansion potential in *vitro*, but still maintain the heterogeneity of the patient tumor. Recent advancements in *in vitro* 3D culture systems such as primary patient-derived organoids can meet this need by providing novel cancer models that better mimic the microenvironment of the originating tumor and exhibit a stable phenotype. A key feature of organoid culture involves embedding cells within a non-defined extracellular matrix that permits the cells to grow in three dimensions into large, complex structures with varying morphologies. These features however, can also make routine quantification of culture health and proliferation challenging. Unlike simpler 2D monolayer cell cultures, organoids do not proliferate as single cells which can make cell counting and viability quantification approaches difficult. The extracellular matrix in which organoids are embedded may also require removal which necessitates additional hands-on processing. Additionally, growth in 3D can interfere with simple visual assessment via brightfield or phase contrast imaging. Here we applied several common approaches for quantification of cell culture health and proliferation to primary patient- derived cancer organoids from multiple tissues, donors and cancer types. Approaches including commercially available kits to quantify metabolism or ATP, as well as the common trypan blue dye exclusion assay were utilized. The results were compared with label free imaging approaches from multiple instrument platforms, which assess growth based on morphological features in brightfield or phase contrast images over time. Additionally, a small-scale toxicity assay was also performed with various chemotherapy drugs to assess the discrimination ability of the assays. Results varied between models, donors, tissues and cancer types. All methods were able to capture long term changes in organoid proliferation, though all faced unique challenges, typically around sample preparation. Traditional in *vitro* assays designed for 2D monolayer cultures could be impeded by the presence of extracellular matrix and had difficulty in penetrating large, multicellular organoids. This resulted in lower signals or higher backgrounds, unless the samples were pre-processed. Imaging based approaches required significant customization and optimization on a per-model basis. Overall, we found that to accurately assess the growth properties of such complex three-dimensional organoid cultures, significant optimization and validation may be required. Depending on the specific application, either imaging based, or cell-based assay approaches may be suitable.

P276/B283

Regulation of ROR1 by Retinoic Acid Induces Differentiation in Neuroblastoma by Modulation of Retinoic Acid Receptor Elements.

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Neuroblastoma is the most common solid neoplasm during childhood. It originates from primitive cells in the sympathetic nervous system that fail to differentiate and propagate rapidly. A common treatment that has been prescribed for over a decade is retinoid therapy (using all-trans retinoic acid (RA)). Treatment with this differentiating agent has been shown to progress the cells from their stem-cell state to a mature neuronal state gaining classical conditions of a neuron, including the repression of proliferation. However, the molecular mechanism behind the action of RA treatment is largely a mystery. Understanding the mechanisms that contribute to both the cancerous properties and

differentiation of neuroblastoma would lead to the discovery of ideal targets for future differentiation therapies. Genomic mining suggests ROR1 activates synaptophysin, a presynaptic regulator that contributes to the formation of synapses. There is also evidence to suggest ROR1 is overexpressed in neuroblastoma but significantly downregulated in mature differentiated neurons. These two functions informed our hypothesis that retinoic acid may modulate ROR1 leading to differentiation and termination of cancerous properties. Immunoblots show that ROR1 levels initially increase and sharply decrease at 96 hours. This is paired with synaptophysin levels sharply increasing at the same time, providing evidence that differentiation ends at 96 hours. Investigation of the ROR1 pathway confirms the downstream mechanism leading to the phosphorylation of β-catenin. Further feedback into the pathway via the MAPK/ERK pathway is seen correlating to the differentiation timescale. To investigate the action of RAR on the expression of ROR1 and its ligand, Wnt5a, ChIP and qPCR were conducted and showed an increase in RAR binding to both genes as well as a sharp expression correlating to its proteomic expression. This data has shown a viable mechanism of action towards neuronal differentiation, as well as repression of neuroblastoma cancer. RA is able to modulate the expression of ROR1 and its ligand to promote differentiation through the expression of synaptophysin and proliferative factors which leads to maturation of the neurons. This research can be further utilized towards targeted therapies in both neuroblastoma, neuronal stem-cell work, and other neuropathologies.

P277/B284

Mechanisms That Impact the Cell Division Axis and Phenotype for Brca1-mutant Mammary Epithelial Cells.

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Background *BRCA1* mutations associate with aberrant mammary progenitor cell numbers and breast cancers that display basal-like features. These features of BRCA1 cancers seeded the concept that BRCA1 exerts proliferative control over normal mammary progenitor cells. In *BRCA1* mutation carriers, progesterone-dependent signaling through RANKL-RANK is a critical mediator of tumor initiation potentially through its promotion of progenitor cell proliferation. We discovered that control of the cell division axis is disturbed by silencing BRCA1 or after one allele was edited to carry a causal *185delAG* mutation. In addition, we observed a propensity for BRCA1-silenced human mammary cells grown as colonies to acquire the features of basal cells. **Underlying hypothesis** BRCA1 mutation disrupts control of the cell division axis in mammary epithelial cells, which cooperates with aberrant RANKL - RANK signaling to produce the altered proliferation and disordered homeostasis observed prior to tumor formation. **Results** When stimulated to divide on an L-pattern, a rounded mitotic cell fixes adhesive structures to the pattern that guide the division axis along the hypotenuse of the shape. We have measured cell division angles in MCF10A parental cells and cells engineered to carry one of 13 distinct BRCA1 variants in one allele in our assay. In BRCA1-mutant cells, however, division angles are oriented only for neutral variants. Cells carrying causal variants (6 of 7) or a VUS do not orient cell division. We observed radiation dose-dependent increases in DNA damage and abnormal cell phenotypes but division angles were not significantly altered. When grown as single cells, MCF10A cells form luminal colonies but BRCA1-silenced cells acquire basal feature. Imaging the colony formation revealed that cell divisions at day 1.5 were not oriented in either treatment. Multi-parameter analysis of individual cells found control-treated cells stayed in proximity with more cell contacts, which correlated with acquisition of oriented cell divisions. To discover pathways, we studied proteomes of BRCA1-silenced

and control-treated colonies. We found that gene ontology terms microtubule and growth regulation, which included regulators of NF-κB signaling, were elevated in BRCA1-silenced colonies. We observed the NF-κB pathway was persistently active in BRCA1-silenced cells. Exposure to RANKL increased the basal-like colonies. TaqMan arrays identified seven gene products elevated in BRCA1- silenced colonies, which we confirmed by IF and immunoblot analysis. **Conclusion** These findings reveal a previously unrecognized consequence of mutant BRCA1 on the cell division axis, post-mitotic integrity and phenotype control in normal human mammary epithelial cells.

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Anti-cancer Cell Properties of *Prunus Africana*Bark Extracts in Mcf-7 Cells Is Cd44 Expression Dependent.

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Prunus africana is a medicinal evergreen tree native to Cameroon and Kenya. Extracts from this tree have been shown to have antimicrobial activity. This activity was dependent on the different part of the tree used to generate the extracts (i.e., leaves, root or bark) as well as the solvents used to prepare them (i.e., acetone, ethanol or methanol). Based on these differential antimicrobial properties, we hypothesize that it is likely that differential anti-cancer cell activities will be found in the different extracts. To test this hypothesis we treated two stably transfected MCF-7-derived breast cancer cell lines; MCF-7/PLXIN (plasmid only control, CD44 negative) and MCF-7/CD44 (pLXIN plasmid expressing CD44 standard, CD44 positive) with Prunus africana bark extracts extracted with aqueous acetone, ethanol or methanol (80% v/v). These extracts were constituted in the same solvents used to prepare the extracts and 10,000 cells per well in 96 well plates were treated in triplicates for 24 h. MTS assays showed higher levels of cell toxicity with ethanol-based and methanol-based extracts compared to acetone-based extracts. This cell toxicity was associated with the expression of CD44 since MCF-7/CD44 cells were more sensitive than MCF-7/PLXIN cells to the extracts. This differential sensitivity indicates that the aqueous ethanol- or methanol bark extracts are targeting the more aggressive CD44-expressing MCF-7-derived cell line.

P279/B286

CHMP6 Negatively Impacts CD147 Levels.

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CD147 (Emmprin, Basigin, BSG) is a single-pass transmembrane glycoprotein that contains two immunoglobulin (Ig)-like domains in the extracellular region. CD147 overexpression has been directly implicated in tumor growth and metastasis. In order for CD147 protein to leave the endoplasmic reticulum and function at the cell surface, it must interact with other proteins. According to the BioGRID, there are currently 203 proteins that potentially interact with CD147, although most of these interactions have no associated functional implications. In order to help explain CD147 overexpression in cancer, we wanted to find new interactants that may impact CD147 levels. We attempted to find relationships between CD147 and various biological processes using a bioinformatics screen. One interesting set of complexes that appeared was the endosomal sorting complex required for transport III (ESCRT-III) machinery. We explored whether a central participant of ESCRT-III, charged multi-vesicular

protein 6 (CHMP6, VPS20), impacts CD147 expression. By western blot, we show that CD147 protein levels are significantly higher in metastatic colorectal cancer HCT116 cells compared to less aggressive HT29 cells. By quantitative RT-PCR, we show that CHMP6 mRNA levels are lower in HCT116 cells compared to HT29 cells. In order to test this inverse relationship between CHMP6 and CD147 levels, we overexpressed CHMP6 in HCT116 cells. By western blot, we observed decreases in both core-glycosylated and fully-glycosylated CD147 levels compared to empty vector controls. By performing fluorescent microscopy on HCT116 cells co-transfected with CD147-GFP and CHMP6-mCherry, we observed partial co-localization of these two proteins. Together, our data indicate that higher levels of CHMP6 correlated with lower CD147 levels.

P280/B287

P53-driven Egfr Recycling and Tumoral Aggressiveness Can Be Counteracted by D-propranolol Involving PKA Activity Down-regulation.

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EGFR regulates processes of proliferation and migration/invasion and is frequently altered in cancer, offering a variety of opportunities as anti-tumoral target. Endocytic trafficking, as the main regulator of EGFR function, can be exploited to counteract EGFR-dependent malignant traits. EGFR recycling with implication in proliferation and invasive migration has been found enhanced in tumoral cells that express gain-of-function (GOF) missense mutations of *TP53*, the most frequent mutations in this otherwise tumor suppressive gene. We are currently studying a PKA pathway that regulates EGFR endocytic trafficking and can be used to arrest both ligand-activated and empty-inactivated EGFR at recycling endosomes. PKA inhibition induces ligand independent EGFR endocytosis and decreases its recycling. As a potential anti-tumoral drug that avoids the toxicity of direct PKA inhibitors we have described the use of D-Propranolol (D-Prop) as an inductor of phosphatidic acid (PA) levels by inhibiting PA hydrolysis. PA activates type 4 phosphodiesterase (PDE4) and subsequently decreases PKA activity mimicking the effects of PKA inhibitors such as H89 on EGFR trafficking. Here we identify proteins involved in PKA-mediated EGFR internalization and assess D-prop effects on cancer cells expressing p53 missense mutations. H89 and D-Prop reduced PKA phosphorylation of PAK4 and GEF-H1 (cytoskeletal regulators) and Rab-Coupling-Protein RCP (Rab11 effector involved in recycling). PAK4 and GEF-H1 silencing inhibited EGFR recycling. H89, D-prop and PKA phosphorylation site mutation S435A of RCP enhanced EGFR interaction with RCP, α5β1 integrin and Rab11, colocalizing at perinuclear recycling endosomes, where the EGFR become arrested with functional consequences upon cell proliferation and invasive migration. In tumoral cells expressing GOF p53 missense mutations, D-Prop inhibited proliferation and invasion, counteracted tumoral growth and increased survival of immunosuppressive mice xenografted with G415-$p53^{R282W}$ gallbladder cancer cells. These results involve PAK4, GEFH1 and RCP as PKA substrates that regulate EGFR endocytic trafficking and demonstrate the anti-tumoral potential of D-prop used to counteract p53-driven EGFR recycling and cancer aggressiveness. (Financed by FONDECYT#1181907 and CONICYT Basal grants AFB170005 and AFB170004)

P281/B288

New Causal Drivers of Estrogen Signaling Revealed by Dense Time Series of Nascent Rna Transcription.

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Mechanistic understanding of Estrogen Receptor (ER) signaling will lead to new therapeutic targets for ER positive breast cancer. To this end, we performed nascent RNA sequencing following estradiol (E2) treatment in MCF7 breast cancer cells every 15 minutes for 6 hours. To date, our study represents the densest time series of estrogen signaling ever reported. We noted three transcriptional waves following E2 treatment: peaks at 30 minutes, 2.5 hours and 5 hours respectively. We noted that the early response was enriched for canonical ER signaling, however mid and late response were enriched for metabolic processes. Because our study was statistically powered with 24 time points, we could use delay-coordinate embedding to look for causal gene interactions. We implicated NME2 as the causal driver of the 5-hour transcriptional wave. Indeed, NME2 and Estrogen Receptor (ER) have been shown to co-localize where NME2 blocks ER effect. Our study represents a new methodology for target discovery in hormone driven cancers.

P282/B289

Phosphofructokinases Axis Controls Glucose-dependent MTORC1 Activation Driven by E2f1.

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Cancer cells rely on mTORC1 activity to coordinate mitogenic signaling with nutrients availability for growth. Based on the metabolic function of E2F1, we hypothesize that glucose catabolism driven by E2F1 could participate on mTORC1 activation. Here, we demonstrate that glucose potentiates E2F1-induced mTORC1 activation by promoting mTORC1 translocation to lysosomes, a process that occurs independently of AMPK activation. We showed that E2F1 regulates glucose metabolism by increasing aerobic glycolysis and identified the PFKFB3 regulatory enzyme as an E2F1-regulated gene important for mTORC1 activation. Furthermore, PFKFB3 and PFK1 were found associated to lysosomes and we demonstrated that modulation of PFKFB3 activity, either by substrate accessibility or expression, regulates the translocation of mTORC1 to lysosomes by direct interaction with Rag B and subsequent mTORC1 activity. Our results support a model where a glycolytic metabolon containing phosphofructokinases transiently interacts with the lysosome acting as a sensor platform for glucose catabolism towards mTORC1 activity.

P283/B290

P53 Activation Attenuates ISR Induced by ETC Inhibition in HCT116 Carcinoma Cell Line.

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The Integrated Stress Response and p53-mediated signaling are two major programs of the cellular stress response. The Integrated Stress Response (ISR) is an adaptive signaling pathway activated by numerous metabolic stresses such as nutrient deprivation, hypoxia, or proteotoxic stress. Transcription factor ATF4 is a key regulator of ISR. The p53 tumor suppressor integrates a wide range of different stimuli and triggers antiproliferative stress responses. The interaction of these two major anti-stress networks has not been well studied. Previously we have shown that p53 activation upon a sustained inhibition of ETC complex III by myxothiazol abolishes the earlier activation of ATF4 and its target genes. The aim of this study is to find out whether p53-dependent repression of the ATF4-mediated transcription is limited to this particular stimulus or represents a common event and to develop approaches to explore the underlying mechanisms. ATF4 was induced by different ISR activators, and levels of mRNAs of ATF4 and its targets (SLC7A11, CHOP, ASNS, TRIB3) in HCT116 cells upon treatment with various p53 inducing agents (doxorubicin, 5-fluorouracil, and nutlin-3a) were assessed using RT-qPCR. We found that treatment with p53 inducing agents caused a decrease in mRNA levels of ATF4 and its targets when ATF4 was not activated or when it was activated by ETC inhibitors piericidin a and myxothiazol, while p21 mRNA level was significantly elevated. Doxorubicin and nutlin-3a also suppressed ATF4-mediated transcription activation during endoplasmic reticulum stress induced by tunicamycin. Thus, we showed that p53-dependent repression of an ATF4-mediated transcription appears to be a common event that follows the induction of endogenous p53 by various stimuli both under normal conditions and upon Integrated Stress Response activation. To confirm the role of p53 in the suppression of ATF4-mediated transcription, we performed the same experiments using HCT116 p53-/- cells. Repression of ATF4-mediated transcription activation upon treatment with 5-FU and nutlin-3a was significantly prevented in the absence of p53. We tested a possible role of p21, which is a known p53 target, in p53-dependent repression of ATF4-mediated transcription in HCT116 p21-/- cells and observed a similar effect. Taken together, these results suggest an important role of the p53/p21-dependent signaling pathway activation in the attenuation of ISR. This work was supported by RFBR grant 18-04-00991 to I.K.

P284/B291

Sur8 Stabilization by PKCa/ddegradation Plays a Role in Transformation, Migration, and Invasion of CRC Cells.

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Scaffold proteins of the mitogen activated protein kinase (MAPK) pathway recruit protein kinase cascades to confer context-specificity to cellular signaling. However, regulatory mechanisms of scaffold proteins are poorly understood. Sur8, a scaffold protein in the Ras-MAPK pathway, is known to be involved in cell transformation and migration, and is increased in human colorectal cancer (CRC) patient tissue. Here we determine that regulation of Sur8 stability mediates transformation and migration of CRC cells. Fibroblast growth factor 2 (FGF2) is identified as an external regulator that stabilizes Sur8. Protein kinase C-alpha and -delta (PKCα/δ) are also identified as specific mediators of FGF2 regulation of Sur8 stability. PKCα/δ phosphorylate Sur8 at Thr-71 and Ser-297, respectively. This phosphorylation is essential for polyubiquitin-dependent degradation of Sur8. Sur8 mutations, which mimic phosphorylation by PKCα/δ and destabilized Sur8, suppress the FGF2-induced transformation and migration of CRC cells. Overall, our findings demonstrate for the first time a regulatory mechanism of Sur8 stability involving cellular transformation and migration in CRC.

P285/B292

G-protein Coupled Receptor 64: a Potential Tumor Suppressor in Endometrial Cancer.

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Endometrial cancer is the most common gynecological cancer. G-protein coupled receptor 64 (GPR64) belongs to a family of adhesion GPCRs and plays an important role for male fertility. However, GPR64 function has not been reported in endometrial cancer. Our objective is to investigate the role of GPR64 in endometrial cancer. We examined the levels of GPR64 in human endometrial cancer tissue by immunohistochemistry analysis. GPR64 levels were significantly lower in 10 of 21 (47.62%) of endometrial carcinoma samples compared to control. To test whether GPR64 has a role of tumor suppressor in endometrial cancer, we used a siRNA loss of function approach in human endometrial adenocarcinoma cell lines. Depletion of *GPR64* by siRNA transfection in Ishikawa and HEC1A cells showed an increase of colony formation ability and cell proliferation. Depletion of *GPR64* also significantly increased the migration and invasion activity of Ishikawa and HEC1A cells. Furthermore, the phosphorylation of AMP-activated protein kinase (AMPK) and expression of *Connexin 43* (*Cx43*), a member of the large family of gap junction proteins, were regulated in *GPR64*-deficient Ishikawa cells. These results suggest that GPR64 play an important role for cell proliferation, migration, and invasion as a tumor suppressor in endometrial cancer.

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TGF-β Induced Alternative Splicing of Tak1 and Implication in EMT and Drug Resistance.

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Transforming growth factor-β (TGF-β) is major inducer of epithelial-to-mesenchymal transition (EMT), which associates with cancer cell metastasis and resistance to chemotherapy and targeted drugs, through both transcriptional and non-transcriptional mechanisms. We previously reported that, in cancer cells, heightened mitogenic signaling allows TGF-β-activated Smad3 to interact with poly(RC) binding protein 1 (PCBP1) and together they regulate many alternative splicing events that favors expression of protein isoforms essential for EMT, cytoskeletal rearrangement, and adherens junction signaling. We now extended these observations to TGF-β-activated kinase 1 (TAK1), which plays an essential role in mediating TGF-β activation of JNK, p38 and nuclear factor kappa B (NF-κB). We found that the exclusion of TGF-β-activated kinase 1 (TAK1) variable exon 12 requires another RNA binding protein, Fox-1 homolog 2 (Rbfox2), which binds intronic sequences in front of exon 12 independently of the Smad3–PCBP1 complex. Functionally, exon 12-excluded TAK1ΔE12 and full-length TAK1FL are distinct. The short isoform TAK1ΔE12 is constitutively active and supports TGF-β-induced EMT and nuclear factor kappa B (NF-κB) signaling, whereas the full-length isoform TAK1FL promotes TGF-β-induced apoptosis. These observations offer a harmonious explanation for how a single TAK1 kinase can mediate the opposing responses of cell survival and apoptosis in response to TGF-β. They also reveal a propensity of the alternatively spliced TAK1 isoform TAK1ΔE12 to cause drug resistance due to its activity in supporting EMT and NF-κB survival signaling. These results uncovered a new mechanism for how TGF-β induces opposing responses to cell survival and apoptosis through TAK1. Furthermore, they provided insights for a new strategy to overcome resistance and to increase sensitivity of tumor cells to cancer chemotherapies and targeted drugs.

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Asap1 Regulates Differentiation in Myoblasts and PAX-FOXO1 Fusion-negative Rhabdomyosarcoma through the Arf GTPase Pathway.

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Rhabdomyosarcoma (RMS), the most frequently diagnosed soft tissue sarcoma in children, is caused by a differentiation defect in skeletal muscle precursor cells. Despite aggressive, multimodal therapies, the prognosis for recurrent PAX-FOXO1 fusion-negative RMS (FN-RMS) remains poor. Inducing differentiation in diseases defined by defective differentiation programs has led to curative therapies in cancers such as acute promyelocytic leukemia. A recent study showed that inducing differentiation slowed tumor growth and extended survival in a xenograft model of FN-RMS, identifying differentiation as a promising therapeutic target in FN-RMS. However, genes associated with differentiation are frequently found to be hijacked by tumor cells and repurposed for pro-invasive programs. Therefore, better understanding of differentiation signaling and its relation to invasion could reveal novel therapeutic opportunities for patients with advanced FN-RMS. We hypothesize that ASAP1, an Arf GTPase-activating protein implicated in differentiation in normal cells and invasion in carcinoma, promotes progression by controlling pro-invasive elements of differentiation signaling through focal adhesion assembly. ASAP1, an Arf GTPase-activating protein (Arf GAP), regulates integrin adhesion complexes, critical regulators of biological processes such as proliferation, migration, and differentiation that are commonly dysregulated in cancer. ASAP1 is overexpressed in several cancers and correlates with increased metastasis and poor patient prognosis but has also been shown to promote differentiation. The mechanisms by which ASAP1 affects cancer progression and differentiation and the relationship between these effects are not yet understood. We found that ASAP1 is overexpressed in FN-RMS. ASAP1 overexpression inhibits proliferation in myoblasts, but not in FN-RMS. Knockdown of ASAP1 inhibits differentiation in both myoblast and FN-RMS cell lines, while overexpression enhances differentiation. Moreover, gene set enrichment analysis shows that myoblast differentiation-associated genes fail to become enriched upon knockdown of ASAP1. Finally, knockdown of Arf1 and Arf5, established binding partners of ASAP1, also block differentiation of FN-RMS cell lines, indicating that ASAP1 may regulate differentiation through its interaction with Arf GTPases. These data support our hypothesis that ASAP1 regulates the continuum of differentiation and invasion in FN-RMS. As a continuing test of the hypothesis, future studies will investigate focal adhesion assembly, dynamics, and signaling, which are processes known to be affected by ASAP1, as a mechanism for ASAP1-mediated regulation of myoblast differentiation.

P288/B295

Metabolic Syndrome Complex Protein 1 Is a Potential Role for Tumor Survival Via Mitochondria Dynamics in Hepatocellular Carcinoma.

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MSCP1 (Metabolic Syndrome Complex Protein 1) released into cytosol by apoptosis signal. After, MSCP1 inhibits phosphorylation at Ser473 of PKBα. PKB is a key molecular in cell signaling relating to cell

survival and an oncogene which induces cell proliferation, growth and anti-apoptosis. Also, MSCP1 is an thioesterase that functions as hydrolysis thioester bonds on medium-chain, long-chain acyl-CoA. Previous study, MSCP1 intensified phosphorylation of PKB in breast cancer and HNSCC(head and neck squamous cell carcinoma). In our study, depending on the cell type, MSCP1 has been shown to influence tumor survival through the regulation of mitochondria dynamics and lipid metabolism in HNSCC. An d we investigated the effect of MSCP1 on hepatocellular carcinoma, because HCC is the most common tumor worldwide. Cell proliferation and migration was decreased in HepG2 cells and Hep3B cells when MSCP1 was overexpressed. Accordingly, we hypothesize that expression of MSCP1 affects tumor survival by regulating mitochondria dynamics and lipid metabolism in HCC.

P289/B296

Multiple Primary Tumors with Differential Drug Sensitivity.

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Localized prostate cancers are uniquely genetically variable, comprising both spatially separate regions and multiple independently evolving clones within each region. The multifocality of prostate cancer is a well-acknowledged problem for diagnosis, and the differential aggressiveness of potentially independent clones remains largely unknown. Further, appropriate prostate cancer staging using multiparametric MRI (mpMRI) and biopsy tissue can be confounded by sampling error. To date, there has been no understanding of whether this variability influences management decisions for localized prostate tumors. Here, we sought to identify the sensitivity and genomic profile of distinct localized tumors from a single patient following systemic intense neoadjuvant androgen deprivation therapy. A high risk prostate cancer patient was enrolled in a Phase 2 study of neoadjuvant androgen deprivation therapy (ADT) plus enzalutamide. mpMRI was performed at baseline, showing a single semi-contiguous lesion encompassing the right apical-mid peripheral zone also extending into the left distal apical peripheral zone. MR/ultrasound-fusion targeted biopsy was performed before undergoing 6 months of intense neoadjuvant ADT. Biopsies of the left- and right-sided tumors showed differing histologies. A second mpMRI was performed following therapy before radical prostatectomy. Laser capture microdissection was performed to isolate pure populations of tumor cells from the pre-treatment biopsy and post-treatment surgical specimen. Whole exome sequencing was performed on microdissected tissues to identify somatic mutations and copy number alterations, which were further used to assess tumor clonal architecture and genomic/phenotypic evolution of treatment resistant tumor. mpMRI and pathology showed near complete response of one tumor and substantial resistance of the other tumor. Histopathological staining and whole exome data highlighted a divergence in the status of tumor suppressor genes implicated in prostate cancer progression, with the non-responding tumor at baseline exhibiting additional alterations absent from the pre-treatment tumor that responded to therapy. Using point mutations as definitive clonal markers, we found that two clonally-independent tumors exhibited intrinsic heterogeneity at baseline which correlated with response or resistance.

P290/B297

Lectin ZG16p Inhibits Proliferation of Human Colorectal Cancer Cells Via Its Carbohydrate-binding Sites.

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Zymogen granule protein 16 (ZG16p) is a soluble 16 kDa protein and is highly expressed in human pancreas, liver and intestine. ZG16p has a β-prism fold structure similar to Jacalin-related lectins (JRLs) and binds to mannose via GG and GXXXD loops which are conserved among JRLs. ZG16p also interacts with negative-charged heparin/heparan sulfate via basic amino acids. ZG16p is highly expressed in the human digestive tract and is secreted into the mucus. It has been reported that the expression of ZG16p is downregulated in colorectal tumor tissue compared with normal tissue and low expression of ZG16p is related with shorter survival of colorectal cancer patients. However, the implication of downregulation of ZG16p in colorectal cancer remains unclear. In this study, to elucidate whether or not ZG16p is involved in the anti-cancer mechanism in the intestinal epithelia, the effect of ZG16p on the proliferation of colorectal cancer cells and patient-derived colorectal tumor organoids was investigated.Overexpression of ZG16p in Caco-2 cells decreased cell growth. Recombinant ZG16p markedly inhibited proliferation of Caco-2, LS174T, HCT116, and HCT15 cells. Caco-2 cell growth was not inhibited by two mutated ZG16p proteins, D151A and M5 (K36A, R37A, R53A, R55A, and R79A) lacking mannose- and heparin-binding activities, respectively. Immunofluorescent cell staining revealed that ZG16p-D151A maintained its binding to the Caco-2 cell surface, whereas ZG16p-M5 failed to bind to the cells. These results suggest that ZG16p interacts with the cell surface via basic amino acids substituted in ZG16p-M5 and inhibits Caco-2 cell proliferation via Asp151. In addition, growth of patient-derived colorectal tumor organoids in a three-dimensional intestinal stem cell system was suppressed by ZG16p but not by ZG16p-M5. Taken together, our findings indicate that ZG16p inhibits the growth of colorectal cancer cells via its carbohydrate-binding sites in *vitro* and *ex vivo*. A novel pathway in cancer cell growth regulation through cell surface carbohydrate chains is suggested.

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Investigating the WDR5 Interaction Network.

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WDR5 is a cellular multitasker, an essential protein involved with histone modifying complexes, transcriptional regulators, mitotic machinery, and more. Because WDR5 is often overexpressed in cancer, understanding and inhibiting WDR5 has therapeutic potential. Key to the functions of WDR5 are mutually exclusive interactions at two binding sites which integrate WDR5 into different cellular processes. One of these binding sites, the Win site, specifically binds arginine-containing motifs of protein binding partners. Integrity of the Win site is necessary for proper function of WDR5, and when the Win site is blocked WDR5 interactions are impaired and a variety of gene expression changes and cellular defects arise. While several direct binding partners of WDR5 are characterized, the specific interactions disrupted by Win site blockade are not known, and it is clear that the current understanding of the WDR5 interactome is not comprehensive. Our work aims to better understand the WDR5 interaction network and determine how these interactions between WDR5 and other proteins influence cellular functions. Recently we and others have been developing small molecules designed to specifically block the Win site. Small molecule inhibition of WDR5 not only carries therapeutic potential but also

allows specific and acute disabling of WDR5 binding sites, enabling basic biological discovery. Our recent work leverages a potent and highly validated small molecule inhibitor of the Win site to identify binding partners of WDR5. Using this inhibitor we performed a SILAC-based quantitative proteomic screen to identify protein binding partners of WDR5 that rely on the Win site. This approach compared Win-site inhibited WDR5 to uninhibited WDR5 and revealed 20 proteins that are specifically displaced from WDR5 by this small molecule. Interestingly, the majority of these 20 proteins have not before been shown to interact with WDR5. Yet every protein from this list that we analyzed by FLAG coimmunoprecipitation validated as interacting with WDR5 in a Win-site dependent manner. These identified WDR5 binding partners set the groundwork for discovering functions of WDR5 that extend beyond the canonical chromatin-linked functions. To focus our study we have narrowed in on a few of these newly identified WDR5-interacting proteins for further characterization of mechanism of binding and functional analysis. These ongoing studies are expanding our understanding of the cellular functions of WDR5. We are learning that WDR5 is an even more versatile and tightly regulated protein than initially grasped. This knowledge is valuable both for understanding how WDR5 is integrated into so many different cellular processes and for informing the development of WDR5 inhibitors as targeted cancer therapy.

P292/B299

Visualizing Native P53 Structures Using Cryo-electron Microscopy.

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The tumor protein TP53, commonly known as p53, is involved in a variety of cellular functions including apoptosis, growth arrest, and DNA repair. For these reasons, it has earned the nickname “the guardian of the genome.” Mutations in the p53 gene are often found in early- stage cancers and may be linked to metastatic processes. Post-translational modifications, such as phosphorylation and ubiquitination, direct p53’s operations in the nucleus through complex structural changes. Surprisingly, little structural information is available for full length p53 as it occurs in human cancer cells. To advance our understanding of p53 architectures in cancer cells, we used cryo-Electron Microscopy (EM) to resolve the tetramer and dimer structures of p53 derived from glioblastoma cells (U87MG line). P53-DNA assemblies were purified from U87MG cells and western blot analyses supported the presence of monomeric wild-type p53 which migrated at approximately 50 kDa. Multiple bands were visible through Comaissee-stained SDS-PAGE analysis within the 50-60 kDa region of the gel. These bands suggested the presence of post-translational modifications to p53. Additional studies revealed the presence of ubiquitinated-p53. Native gel analysis confirmed the migration of p53 tetramers that migrated ~220 kDa along with dimers that migrated ~110 kDa. Cryo-EM images confirmed a mixture of dimers and tetramers present in the preparation. We used the RELION software package to perform 3D classification and refinement procedures to produce multiple EM structures. The resulting EM maps accomodated the models of p53 and were consistent with experimentally-determined class averages calculated from the overall particle populations. Reconstructions were refined to close to 10 Å according to 0.5 Fourier shell correlation criteria. The p53 tetramer structure suggested that a continuous presence of density within the model was an intact DNA strand. Dimer structures also had extra density where two ubiquitin models were placed in a biologically-relevant manner. These features represent captured p53 assemblies performing one of their most essential functions related to DNA repair while receiving post-translational modifications. Overall, we posit that Cryo-EM can be used as a new

technique to understand medically-relevant protein targets to advance our understanding disease-related protein assemblies.

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Tissue Architectural Cues Drive Organ Targeting of Tumor Cells in Zebrafish.

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Clinical patterns of cancer metastasis are non-random, with certain types of cancers preferentially metastasizing to certain secondary organs in a process termed organotropism. Genetic profiling of cancer cell lines that metastasize to the brain or bone marrow in mice have elucidated important genetic drivers of organ colonization. However, the impact of the microenvironment on organ selectivity during metastasis is less understood, particularly because of a lack of models to concurrently visualize the early stages of metastasis in multiple tissues within the same animal. Here, we used a zebrafish xenograft metastasis model to determine drivers of organ selectivity during early metastasis. The zebrafish is amenable to imaging at single-cell resolution in multiple organs in addition to possessing blood vessels on the scale of human capillaries, a brain with structural and cellular similarity to the mammalian brain, and hematopoietic tissue in the caudal vein plexus (CVP) analogous to bone marrow. Breast cancer cells that target specific murine organs (brain and bone marrow) ultimately colonized analogous tissues (brain and hematopoietic tissue in the CVP) in larval zebrafish after injection to the circulation. This pattern was conserved across human MDA-MB-231 brain- and bone marrow-targeting subclones (231BR and 231BO, respectively), brain-seeking human Bt474m1 cells, and murine 4T1 bone marrow- and brain-seeking cells. Initial cell dissemination was non-random, with more cells arresting in the complex vasculature of the CVP than the brain, but was independent of cell type, with brain- and bone marrow-targeting cells arresting at identical rates in a given organ. Similar results were obtained with inert beads and upon knockdown of β1 integrin in 231 cells. Using a combination of live-cell imaging, mechanical mapping of tissue properties, confined cell migration assays, and quantification of vascular architecture, we determined that vessel topography was the key determinant of initial cell arrest. Following arrest, a significantly higher fraction of 231BO compared to 231BR cells extravasated in the CVP, leading to the observed organ targeting. Mass spectrometry of 231BO and 231BR cells indicated that pathways involving β1 integrin were upregulated in bone marrow-targeting cells, while myosin 1B was overexpressed in brain-targeting cells. Knockdown of integrin β1 reduced extravasation in the CVP and redirected cell targeting toward the brain. Conversely, knockdown of myosin 1B decreased extravasation in the brain and lead to greater targeting of the CVP. In conclusion, while non-random targeting during initial dissemination was driven by differences in organ vasculature, organ selectivity was dependent on extravasation ability following cell arrest.

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Tuning Cell Contractility and Vinculin Localization at Cell Junctions Is Required for Basal Epithelial Cell Extrusion.

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Metastasis is the main cause of cancer-related deaths. How these cancer cell alterations evolve within tightly regulated tissue remains elusive. We show that an oncogenic mammary epithelial cell surrounded by normal cells could in *vivo* basally extrude and disseminate at very early stage before the formation of a primary tumor. The key polarity protein atypical PKC iota (aPKCi) overexpression is crucial for oncogenic basal cell extrusion in *vivo*, a new mechanism for early breast tumor cell dissemination. Moreover, we highlight the importance of the different mechanical properties between the oncogenic aPKCi overexpressing cells and the surrounded normal cells associated with the decrease of vinculin at the cell junction which triggers cell segregation, first step promoting and controling the direction of cell extrusion. In fact, by combining biophysical approaches, we show an increase in cell tension at the interface between aPKCi+ and WT cells dependent on myosin II activity, associated with a relocation of vinculin from cell-cell junctions to focal adhesions in aPKCi+ cells, leading to the acquisition of pro-migratory features in these oncogenic cells. We identify aPKCi and vinculin as new regulators of cell segregation and propose that a balance between cell contractility and cell-cell adhesion at the interface between normal and oncogenic aPKCi+ cells is crucial for promoting basal cell extrusion. We anticipate that this mechanism may be conserved in other carcinomas, promoting early cancer cell dissemination.

P295/B302

Spastin-Pin1 Interaction in Glioblastoma Motility.

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Glioblastoma multiforme (GBM) is the most malignant type of glioma. Since GBM has high invasion-migration capacity, it can recur rapidly after surgery. The capacity of tumor cells to migrate is attained by the formation of invadopodia that are formed through actin polymerization and further growth requires microtubule (MT) fragments which are cut into shorter pieces by MT-severing proteins from long MTs. While the expression level of the MT-severing protein Spastin is at the basal level in glial cells, its expression is increased in GBM cells. Since Spastin has been shown to localize in cell cortex where the actin is intensely located to regulate cell motility-migration, it might have an important role in the invasion of tumors. Dysregulation of proline-mediated phosphorylation mechanisms intervened by protein kinases such as cyclin-dependent kinases (CDKs) is a common phenomenon observed in cancer. Pin1 is an enzyme that leads to conformational and functional changes on its targets by isomerization of p-Ser/Thr-Pro residues. Theretofore, studies demonstrated that Pin1 may contribute to invasion mechanism by affecting proteins that trigger cell motility. Spastin has numerous Ser/Thr-Pro motifs such as T292-Pro, and T303-Pro on its microtubule binding domain (MBD). Thereupon, we suggested that the reason for the co-localization of Spastin with actin in GBM is that Pin1 may interfere with the binding of Spastin to MTs by interacting with p-Thr-Pro motifs located on the MBD. To examine whether MBD-Pin1 interaction directly affects the localization of Spastin, two different vectors containing either full-length Spastin or Spastin-MBD were cloned to identify this interaction. Through site-directed mutagenesis, Asp and Ala mutations were introduced into both Thr292 and Thr303 sites to mimic constitutively

phosphorylated and phosphorylation inhibited form of Spastin, respectively. Then, the interaction of Pin1 with wild-type and two different mutant forms of full-length Spastin or Spastin-MBD was investigated with the co-immunoprecipitation (co-IP) assay. The co-IP analysis of Spastin-MBD with Pin1 showed that Pin1 interacts with only phosphorylated form of Spastin-MBD. The co-IP between full-length Spastin and Pin1 indicated that interaction between Ala mutant of Spastin with Pin1 continues through Thr-Pro motifs located outside of the MBD, a significant increase was observed in Pin1 interaction with the Asp mutant of Spastin. All these results show that although not alone, Thr-Pro motifs on the MBD play an active role in Spastin-Pin1 interaction. To understand whether the conformational change of Spastin-MBD through Pin1 interaction is the cause of the orientation of Spastin from MTs to actins in GBM, immunocytochemical analysis will be examined in future studies.

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Blocking Interaction between Talin2 and β1-integrin Inhibits Matrix Metalopeptidase 9 Secretion in Breast Cancer Cells.

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Talins and integrins are key proteins in several cellular processes, including cell migration, adhesion and metastasis. We demonstrated in our previous work that in breast cancer interaction between talin2 and β1-integrin is crucial for matrix degradation, traction force generation, cell invasion (Qi et al., 2016), tumorigenesis and metastasis (Li et al., 2017). However, the molecular mechanism connecting these phenomena with talin2 and β1-integrin interaction still remains unclear. Our current research concentrates on talin2 - β1-integrin-mediated molecular mechanism of secretion matrix metallopeptidase 9 (MMP-9), that leads to extracellular matrix (ECM) degradation in breast cancer cells. We show that only fully functional interplay between β1-integrin and talin2 can provide docking site for MMP-9 containing vesicles, which is required for MMPs secretion, and consequently, to ECM degradation. Our studies, based on talin2-knockout MDA-MB-231 breast cancer cell line, indicated that depletion of talin2 inhibits secretion of MMP-9. Moreover, a single mutation in talin2 protein in Ser339, leading to a significant decrease in talin2 - β1-integrin affinity, has a similar result. Furthermore, we investigated the fate of MMP-9 in these cells, finding its accumulation in enlarged endosomes and lysosomes. These result suggest that talin2 - β1-integrin complex mediates secretion MMP-9, and a disorder in that interaction disturbs the MMP-9 trafficking process leading to overflow of a lysosomal degradation process. Li, L, Li, X, Qi, L, Rychahou, P, Jafari, N, and Huang, C (2017). The role of talin2 in breast cancer tumorigenesis and metastasis. Oncotarget 8, 106876-106887. Qi, L, Jafari, N, Li, X, Chen, Z, Li, L, Hytönen, VP, Goult, BT, Zhan, C-G, and Huang, C (2016). Talin2-mediated traction force drives matrix degradation and cell invasion. J Cell Sci 129, 3661-3674. This work is supported by a grant from National Institutes of Health Grants R01 GM122994 (to CH), and a subvention from the Polish Ministry of Science and Higher Education 2019-N17/MNS/000006 (to ZB).

P297/B304

Alteration of Pituitary Tumor Transforming Gene 1 (pttg 1) by Microrna Regulates Migration Ability of Human Oral Squamous Cell Carcinoma Via Mmp-2 and Mmp-9 Expression.

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Human pituitary tumor transforming gene 1 (PTTG1), which is an identified proto-oncogene in pituitary tumor, highly express in the tissues with proliferating activity as well as several cancers. In the previous report, we demonstrated that PTTG1 involve in migration ability of oral SCC cell using down-regulation of PTTG1 by siRNA. However, the regulation mechanism of PTTG1 in oral SCC and PTTG1 targeted microRNA capable of regulation of their expression are still unclear. The objectives of this study are to analyze the expression of PTTG1 in oral SCC cell lines (YD-10B and YD-15) and demonstrate the effect of PTTG1 on oral SCC cell lines for the migration by PTTG1 siRNA and microRNA treatment targeted to PTTG1. In addition, the effect of PTTG1 by microRNA-186 (miR-186) and -655 (miR-655) targets to PTTG1 on migration ability of oral SCC cells was evaluated. Western blot, migration assay, and zymography were performed to evaluate the effects of PTTG1 on the expression of MMP-2/-9 and migration activity. The expression of PTTG1 in YD-10B cell line was stronger than that of YD-15 cell line. The expression of PTTG1 in both cell lines was significantly decreased without changes of their characterization in oral SCC cell lines by siRNA-PTTG1 treatment ($p<0.001$). Also, the migration abilities of both YD-10B and YD-15 were significantly decreased through decreasing MMP-2 and MMP-9 expression after siRNA-PTTG1 ($p<0.001$). Interestingly, down-regulated PTTG1 expression by siRNA differently induces to change the expressions integrin and Rho family in YD-10B and YD-15 cell lines. Furthermore, PTTG1-targeted miR-186 and miR-655 directly regulate the expression of PTTG1 and control the migration ability of oral SCC cells through regulation of PTTG1 expression ($p<0.001$). These results suggest that the alteration of PTTG1 by miR-186 and miR-655 could be controlled migration of human oral SCC cells via MMP-2 and MMP-9 expression. Therefore, these findings provide useful guideline for the migration mechanism of oral SCC by regulation of PTTG1 expression. This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2019R1I1A3A01057886)

P298/B305

Stimulation of Glioblastoma Motility by Activated Macrophages Paracrine Signaling Involves Changes in Cytoskeletal Dynamics and Cell Morphology.

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Glioblastoma multiforme (GBM) is one of the most common and deadliest primary malignant cancers of the central nervous system. The main challenge in treating GBM remains their high ability to infiltrate healthy brain tissues. GBM growth and invasion can be modulated by communication between tumor cells and their microenvironment. Microglia and infiltrating macrophages constitute up to one third of GBM tumors. We thus investigated the mechanisms by which macrophages stimulate GBM cell invasion. We first examined the effects of activated M1-type THP-1 macrophages paracrine signaling on cell motility and adhesion of U-87 MG and T98G cancer cells in *vitro*. Our results show that treatment with activated THP-1 conditioned media (CM) increases 2D cell motility and downregulates adhesion to collagen as compared to the control. Interestingly, the CM induced morphological changes in both cell lines including the formation of membrane protrusions and membrane ruffles as well as an increase in

cell-cell connecting tunnel-like structures. Quantitatively, the surface area of treated cells was around two folds larger than that of control cells, and this observed phenotype was reversed upon replacement of the CM. Further analysis by pull down assays and Förster resonance energy transfer (FRET) demonstrated a role for Rho GTPase activation in the GBM cancer cells response to treatment with CM from the activated macrophages. Altogether the data indicates that M1-type activated macrophages stimulate GBM motility in a paracrine manner by upregulating key structures involved in cell motility and invasion. Further work is still warranted to determine the molecular targets implicated in the macrophages-GBM paracrine signaling.

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Stard13 Is a Tumor Suppressor in Lung Adenocarcinoma Cells That Differentially Regulates Cell Migration and Invasion through Rho Gtpases.

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Lung cancer is the second most commonly occurring cancer among both men and women. The ability to metastasize and spread to distant locations renders the tumor more aggressive. Metastasis depends on the cell's migratory ability, which requires the timely and coordinated regulation of the actin cytoskeleton. Members of the Rho subfamily of small GTP-binding proteins (GTPases) play a central role in the regulation of the actin cytoskeleton and in cancer cell migration and metastasis. The switch between active GTP-bound and inactive GDP-bound state is regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine-nucleotide dissociation inhibitors (GDIs). Here we studied the role of the RhoA/Cdc42 GAP StarD13, a previously described tumor suppressor, in malignancy, migration and invasion of lung the cancer cells A549. The decrease in the level of expression of this Rho-GAP in tumor tissues compared to non-patients suggested it is indeed a tumor suppressor in lung cancer. Knocking down StarD13 through transfecting A549 cells with StarD13 siRNA, led to an increase in cell viability, seen by WST, cell cycle analysis and an nexin staining. StarD13 however was required for cell migration. In fact, StarD13 knockdown resulted in an inhibition of cell motility by wound healing and time lapse random migration.StarD13 siRNA-transfected cells were not able to detach their tail and move forward. This was due to the constitutive activation of RhoA seen by pull down assay and FRET. StarD13, however, was found to be an invasion suppressor in an invasion assay. This was due to its inhibitory role of Cdc42-mediated invadopodia. Indeed when StarD13 was knocked down, Cdc42 activation increased and invadopodia formation seen through TKS4 staining increased as well. This was reversed with Cdc42 knockdown. In conclusion, StarD13 has different effect on lung cancer cell migration and invasion through its regulation of Rho GTPases.

P300/B307

Ror1 Signaling through Dvl and Rif Promotes Invasion of Lung Adenocarcinoma Cells.

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Ror1 receptor tyrosine kinase mediates Wnt5a-induced signaling, thereby regulating tissue-/organo-genesis during embryonic development. Ror1 is also expressed highly in various types of cancer cells and promotes their proliferation, invasion, and metastasis, depending on their cell types. However, it is unclear whether Ror1 regulates these processes by acting as a Wnt5a receptor in cancer cells. Here, we

show that lung adenocarcinoma PC-9 cells express both Wnt5a and Ror1, leading to constitutively activated Wnt5a-Ror1-Dvl signaling, which is required for their invasiveness. We also show that Ror1 induces formation of filopodia through the Rho-family small GTPase, Rif (rho in filopodia, also known as RhoF). Interestingly, Rif-mediated filopodia formation is highly polarized and associated with degradation of Matrigel during invasion of PC-9 cells. In fact, suppressed expression of Rif in PC-9 cells inhibits their invasiveness. We will discuss how Rif-mediated filopodia formation can associate with Wnt5a-Ror1-Dvl signaling to promote cancer cell invasion.

P301/B308

The Roles of Actin Regulators in Transendothelial Migration of Uveal Melanoma Cells.

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Uveal melanomas (UM) are cancerous tumors arising from melanocytes in the pigmented layers of the eye - the iris, ciliary bodies, and choroid - collectively called the uvea. UM cells can only spread hematogenously through the bloodstream, but they enter the bloodstream readily: nearly half of UM patients die of metastatic disease; and circulating UM cells are detectable in patients who never develop metastatic disease. We have focused on extravasation, specifically transendothelial migration (TEM), of UM cells as the essential first step in metastatic progression. We have shown that UM cells execute TEM via a stepwise process: UM cells adhere to the luminal surface of endothelial cells; they intercalate into the endothelial monolayer and remain intercalated, often for hours; they then extend actin-rich protrusions beneath the monolayer, and then detach and migrate away under the endothelial monolayer. Genetically: UM tumors are driven by oncogenic mutations in *GNAQ* or *GNA11* in 90% of cases; and tumors that subsequently lose the tumor suppressor *BAP1* have a >80% chance of metastasizing. G-alpha-q/11 activates TRIO, a guanine nucleotide exchange factor (GEF) for both RhoA and Rac1 small GTPases. BAP1 regulates the transcription of Rac1, cortactin, and several RhoA GTPase activating proteins (GAPs). We are using specific inhibitors to define the roles of Rho and Rac activation in TEM of GNAQ(Q209L), BAP1 wt UM cells and CRISPRs to link their effects on TEM to BAP1 loss. We are also using RhoA and Rac1 FRET biosensors to quantify the cellular localization of activation of each during TEM. Our findings suggest that the G-alpha-q/11 and BAP1 pathways intersect at these key regulators of motility and migration to drive TEM in UM cells.

P302/B309

Ccl18-elicited Phosphorylation of Acapin Steers Breast Cancer Cell Migration and Metastasis.

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Tumor metastasis represents the main causes of cancer-related death. Our studies show that ARF6 GTPase-activating protein ACAP4 regulates directional breast cancer cell migration in response to chemokine CCL18 (Chen, 2011. *Cancer Cell*; Song, 2018. *JMCB*). Here, we report a critical mechanism of action underlying Akt signaling in promoting breast cancer metastasis. Using functional proteomics screen, we identified a novel ARF6-ACAP4 signaling regulator, Acapin, which inhibits the GAP activity of ACAP4 to promote ARF6-GTP in *vitro*. CCL18 stimulation elicited Akt signaling which recruits Acapin to the lamellipodium membrane via phosphorylation of Acapin. Importantly, Akt-induced phosphorylation of Acapin promotes the binding of Acapin to ACAP4 which elevates ARF6-GTP level. Acapin was also found to be required for efficient CCL18-Akt stimulation of directional cell migration *in vitro* and breast

cancer metastasis in xenografts. Significantly, elevated level of phopho-Acapin correlates with increased levels of phospho-Akt and ARF6-GTP as well as poor prognosis in patients with advanced/metastatic breast cancer. Our results demonstrate that Acapin relays CCL18-elicited Akt signaling cascade and is a key determinant in chemokine-stimulated breast cancer metastasis.

P303/B310

Chromatin Remodeling Drives Metastatic Potential of Bladder Cancer Cell Lines.

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Given the difficulty to treat metastatic bladder cancer (BC), it is critical to identify tumors with metastatic potential. Previous studies suggested that epigenetic alterations play essential roles in changing transcription during cancer progression. However, how BC cells establish an altered epigenetic landscape and the relationship to metastasis remains mostly unknown. Thus, we investigated how the genome-wide chromatin landscape evolves during BC progression by surveying genome-wide DNase hypersensitivity sites (DHS) in 16 BC cell lines with various metastatic potential as a readout of epigenetic activities. The DHS profiles showed large-scale alterations with recurrent loss of chromatin accessibility during metastatic progression, mainly affecting non-promoter DHS. Most of the lost DHS were unique to normal urothelial cells indicating the loss of cell identity. Ingenuity Pathway an alysis identified SMARCA4 as the top upstream transcription regulator of the potential target genes regulated by the recurrently lost/gained DHS in metastatic BC. A novel homozygous SMARCA4 deletion of several exons was found in metastatic cells of the T24 lineage. Suppressed SMARCA4 expression was also evident in two other metastatic lineage cells, UMUC3/LUL2. Although we found no SMARCA4 alteration in other metastatic cells - 253J, BV and PDX, other SWI/SNF subunits were affected. 253J/BV and PDX carried homozygous nonsense mutations in SMARCB1 and ARID1A, respectively. In contrast, no homozygous alterations in 12 other components of SWI/SNF complexes were found in 6/6 non-metastatic BC cell lines. In the TCGA database, of the 12 genes that encode components of the SWI/SNF complex, seven genes had low expression or truncating mutations in at least two bladder tumors, and some of SWI/SNF complex subunits showed a trend for association with reduced PFS individually or in combination of all three genes ($p < 0.01$). Low expression/protein-truncating mutations in the three genes were significantly more common in stage 3-4 tumors compared to stage 1-2 tumors ($p < 0.01$). In summary, our study showed that loss of function alterations in SWI/SNF subunits is associated with large-scale recurrent loss of DHS in metastatic BC cell lines and poor clinical outcomes/higher tumor stage in TCGA BC patients, confirming the importance of altered genome structure and chromatin remodelers for the metastatic potential of BC.

P304/B311

3D Genome Organization Changes Associated with Melanoma Confined Migration.

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To migrate through constrictions of the extracellular matrix or endothelial lining, cells must dramatically squish and squeeze their nuclei, causing major deformations. The size and stiffness of the nucleus constitutes a rate limiting factor during migration. Despite the detailed characterization of the 3D genome organization, it is not yet known whether a specific organization influences or is affected by

constricted migration. Using melanoma (A375) as a model of metastasis, we have identified an inherent heterogeneity in migratory competence of these cells. Through sequential rounds of constricted migration, we have been able to isolate subpopulations of A375 that are poor migratory (A375-NM) and highly migratory (A375-M). Interestingly, as A375-M cells progressed through rounds of constricted migration, they displayed a stable increase in migration efficiency suggesting inherent changes in chromatin organization and gene expression. Using Hi-C to measure 3D genome structure, we found that A375-M exhibit a distinct genome organization with specific regions of the genome that switch compartments when compared to A375-NM. Some of these changed regions correspond to genes correlated with metastatic progression. By integrating imaging and multi omic data analysis, we are able to characterize the interplay between gene expression, epigenetics and global changes in genome organization during confined migration. These observations reveal a previously unidentified relationship between the 3D organization of the genome and ability of melanoma cells to migrate through constricted spaces.

P305/B312

Overexpression of Nuclear Envelope Proteins in Metastatic Melanoma Promote Loss of Nuclear Envelope Integrity during Confinement.

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Metastatic melanoma is an aggressive disease, characterized by its high level of mutational burden, resistance to traditional chemotherapies, and rapid metastasis, the primary cause of melanoma mortality. To metastasize to a distal site, cells must migrate through tissues which impose physical constraints, and regions of high confinement, resulting in nuclear deformation and loss of nuclear envelope (NE) integrity. This exposes chromatin to cytosolic DNases, generating double strand breaks, that when repaired improperly result in heritable genomic aberrations. We hypothesize that during metastatic progression, the expression levels of genes encoding NE proteins are altered, promoting nuclear deformability and envelope fragility, endowing cells with a greater ability to migrate in confinement and driving genomic plasticity. To determine the changes in NE gene expression during disease progression, we compared published RNA-seq transcriptomic data sets from patient tumor samples of metastatic BRAF V600E melanoma and benign nevi, metastatic melanoma cell lines, and primary human melanocytes, focusing on a subset of NE proteins transcriptionally upregulated in metastatic disease. To determine their role in modulating nuclear integrity during confinement, we performed a targeted siRNA-based screen of 10 genes and assayed for nuclear fragility using a pressure-driven PDMS confinement device to rapidly and precisely confine cells bearing fluorescent markers of histones, nuclear membranes, DNA exposure to the cytosol, and a diffusible nucleoplasmic marker. We found that under 3 μm confinement 35.4% of melanoma cell line 1205Lu exhibit DNA exposure to the cytosol, compared to only 10.1% of melanocytes. Reduction of NE proteins Lamin B2 and Lamin B Receptor (LBR) in 1205Lu cells reduced DNA exposure to the cytosol to 8.2% and 5.7% respectively. To determine if nuclear mechanics were affected, we utilized atomic force microscopy combined with a laser scanning confocal to analyze nuclear stiffness. We found that melanocytes have a dramatically stiffer nucleus compared to metastatic melanoma lines, however overexpression of LBR was sufficient to reduce nuclear stiffness in melanocytes. These results show that upregulation of specific NE proteins in metastatic melanoma promote confinement-induced NE fragility, and can play a role in altering nuclear

mechanical properties, potentially promoting genetic heterogeneity in metastatic melanoma during migration in confined microenvironments.

P306/B313

Protumorigenic Mechanisms of LCN2 Action within the Premetastatic Lung.

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Mortality for patients with metastatic breast cancer is over 60% at five years post-diagnosis. Thus, there is a critical and urgent need to identify targetable mechanisms that support breast cancer cell dissemination to and expansion within the metastatic niche. In this regard, previous work has established mechanisms by which cancer cells prime these premetastatic tissues, and we recently established a tumor cell-free syngeneic in *vivo* breast cancer model for characterizing tumor cell secretome-mediated reprogramming of premetastatic tissues. Using this model, we reported that secretomes from metastatic breast cancer cells, enriched for a prognostically unfavorable lipocalin 2 (LCN2) axis, induced anti-inflammatory MSC actions and a tumor-supportive premetastatic lung. Interestingly, previous work has demonstrated that LCN2 expression in hypoxic murine kidneys requires deoxyhypusine synthase (DHPS)-dependent hypusination/activation of the eukaryotic translation initiation factor 5A (eIF5A1/2) - an established tumorigenic node. Thus, we asked whether eIF5A1/2 activity is necessary for LCN2 expression in metastatic breast cancer cells. Notably, LCN2 expression in the metastatic triple-negative Py230 breast cancer cells was abrogated following treatment with a DHPS inhibitor. To further characterize potential LCN2 regulatory and/or effector pathways, we analyzed the prognostic significance for a focused set of common eIF5A1/2 and tumor heterogeneity markers. Of these proteins, only genomic alterations in LCN2 or HSPA5 (GRP78 or BiP) genes significantly correlated with decreased median survival of breast cancer patients. In combination with our ongoing studies aimed at identifying Py230 secretome-induced transcriptome changes within the premetastatic lung, these data promise to identify novel targetable mechanisms by which primary breast tumors communicate with the premetastatic niche to promote breast cancer progression and patient mortality.

P307/B314

The Eif5a1/2 Pathway Mediates Tgfb/fn1-induced Metastasis and Associates with Prognostically Unfavorable Sox2/tp53 Genomic Alterations in Breast Cancer Patients.

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Metastatic breast cancers carry a 5-year survival prognosis of less than 20%. Thus, it is highly desirable to identify therapeutic strategies that specifically target both primary and metastatic tumors. It is generally accepted that epithelial-mesenchymal transition (EMT) is an important regulator of therapy responsiveness, contributing to intratumoral heterogeneity and systemic dissemination in solid tumor types such as breast cancer. Since we have previously reported that posttranslational hypusination of eukaryotic Initiation Factor 5A (eIF5A1/2) is required for Pseudopodium-Enriched Atypical Kinase One (PEAK1) translation and PEAK1 mediates malignant phenotypes of Transforming Growth Factor β (TGFβ) signaling, we hypothesized that TGFβ may directly regulate eIF5A1/2 activity during EMT and that targeting this pathway may effectively impair metastatic breast cancer cells. We show evidence of an active eIF5A/PEAK1 pathway in undifferentiated, mesenchymal breast cancer tissue. Notably, inhibition

of eIF5A hypusination blocks PEAK1 translation, cell viability, and TGFβ/fibronectin-induced EMT and metastasis of breast cancer cells. Further, assessment of subcellular eIF5A1/2 in response to inhibitors of the hypusination pathway revealed cell line-dependent patterns that suggest eIF5A1/2 functions to drive intercellular heterogeneity. Using a gene set defined by PEAK1-induced EMT and eIF5A1/2 pathway markers, we generated an instructive interactome containing 12 nodes represented in the initial gene set and an additional 74 interacting nodes. Bioinformatic analyses identified SOX2 and PT53 as DHPS interactors within this PEAK1-eIF5A1/2 interactome that significantly contribute to diminished patient survival when genomically altered in combination with DHPS. Taken together, these results suggest that combinatorial therapies targeting DHPS and PEAK1-SOX2/TP53 axes may reduce intratumoral heterogeneity and improve breast cancer outcomes.

P308/B315

Dietary Soy Isoflavone Metabolite Equol Regulates Translation Initiation Factors to Promote Metastatic Breast Cancer Progression.

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Protein synthesis initiation plays an important role in post transcriptional regulation. The eukaryotic initiation factor 4G1 (eIF4G1) is known to be overexpressed in breast cancer cells, and thus, to promote the expression of pro-cancer molecules. In previous studies, we reported that in a breast cancer metastasis mouse model, daidzein increased breast cancer progression and that in vitro, its metabolite equol is primarily responsible for daidzein's cancer promoting effects via upregulation of eIF4G1. Also, a polysome profile study demonstrated that equol upregulated eIF4G-depepndent non-canonical protein synthesis of a number of pro-cancer molecules. Since downregulation of eIF4G did not completely abrogate the effects of equol in breast cancer cells, we asked the question whether equol regulates other initiation factors such as DAP5, a homolog of eIF4G1. To validate the effect of equol in vivo and to investigate its effect on protein synthesis initiation, we created cell lines with stable knockdown of eIF4G1 via a Tetracycline-inducible promoter driving a short hairpin RNA (shRNA) targeting eIF4G1. Next, the MDA-MB-435 metastatic cancer cells expressing control or eIF4G1 knockdown were used to create mammary fat pad tumors in immunocompromised SCID mice. Doxycycline (50 mg/kg BW) was administered to selected groups via oral gavage to induce the expression of sh-eIF4G1 after tumor establishment. Also, Vehicle (90% corn oil, 10% ethanol) and equol (10 mg/kg BW) were given via oral gavage. All treatments were administered 3X week for a total of 65 days. Data shows that equol increases mammary tumor growth when compared to the vehicle. Also, western blot analysis of the tumors show that Dap 5 was expressed even after eIF4G1 knockdown. This was validated also in-vitro. This data suggests that there may be a compensation effect by DAP5 to protein synthesis initiation when eIF4G1 is knocked down in metastatic cancer cells. In conclusion, eIF4G1 knockdown abolishes the effect of equol in cancer progression. This validates our hypothesis that equol increases breast cancer progression via regulation of protein synthesis initiation. Future studies will determine the effect of equol in metastatic cancer cells following knockdown of the homologous protein synthesis initiation factors eIF4G1 and Dap5.

P309/B316

Rab13 and Net1 Mrna Localization and Roles during Collective Invasion.

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Targeting of mRNAs to specific subcellular regions, and the ensuing spatial regulation of protein production, is important for various processes, including migration, epithelial cell polarity, and neuronal function. Our lab has described a pathway of mRNA localization that targets a group of ~80 mRNAs to cell protrusions. Disrupting this localization mechanism impairs single-cell migration on 2-dimensional surfaces as well as invasion through a 3-dimensional matrix. Among these protrusion-localized mRNAs, we focus on two, which encode Rab13, a small GTPase with a role in intracellular membrane trafficking, and Net1, a guanine nucleotide exchange factor (GEF) for the RhoA GTPase. Here, we explore the role of Rab13 and Net1 RNA localization in collectively invading cancer cells, characterized by the maintenance of cell-cell contacts. We recapitulate collective invasion using MDA-MB-231 breast cancer spheroids, which become invasive upon withdrawal of serum. We performed RNA FISH to assess the distributions of Rab13 and Net1 RNAs in invading spheroids, and developed a novel method of quantifying and statistically analyzing the distributions. Interestingly, we find that both the Rab13 and Net1 mRNAs localize to the front edge of leader cells in invasive strands. To disrupt this distribution, we employed a method that we recently developed. This method relies on antisense oligonucleotides that specifically target localization elements contained within the 3' UTRs of the Rab13 and Net1 RNAs. Indeed this approach can specifically prevent the polarized distribution of the RNA being targeted (either Rab13 or Net1) within the leader cells of invasive strands. Interfering with this distribution also causes a decrease in spheroid invasiveness, suggesting that the distribution of the Rab13 and Net1 RNAs plays an important functional role in the ability of spheroids to invade. We are utilizing these techniques to investigate the mechanisms underlying Rab13 and Net1 RNA localization and to assess the functional effects of local GTPase regulation during collective invasion of cancer cells. Moving forward, we are also interested in testing if delivery of morpholinos that prevent Rab13 or Net1 RNA localization can reduce metastasis in in vivo models.

P310/B317

The Effect of PTHrP on the Alcohol-induced Proliferation, Invasion and Migration of Mcf-7 and Mda-231 Cells.

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Epidemiological and experimental studies have shown a link between moderate alcohol consumption and an increased risk of breast cancer in women. In estrogen receptor positive cells (ER+) alcohol affects estrogen signaling which leads to an increase in cell proliferation, invasion and migration. However, the effects of alcohol on estrogen receptor negative cells are not as well understood. Parathyroid hormone related protein (PTHrP) is a paracrine growth factor that has been shown to be a potential regulator of estrogens' effects in the mammary gland. Overexpression of PTHrP in the mammary gland inhibits estrogen induced growth of the mammary gland during puberty. In the present study we the effects of PTHrP on the alcohol induced cellular responses of ER positive (MCF-7) and ER negative (MDA-MB-231) breast cancer cells were analyzed. Results showed that alcohol lead to an increase in the invasion and migration of both cell types. However, alcohol treatment increased cellular proliferation only in ER+ MCF-7 cells. Pre-treatment with PTHrP inhibited the alcohol induced proliferation of MCF-7 cells. Pre-treatments with PTHrP did not alter invasion or migration of either cell type. Together these results

suggest that alcohol induce cellular proliferation, but not invasion and migration, is due in part to the activation of estrogen receptor signaling and that this activation can be inhibited by PTHrP.

P311/B318

Ultrastructural an alysis of Inflammatory Breast Cancer Cell Clusters in an Environment Mechanically Mimicking the Lymph Vascular System.

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Background: Inflammatory breast cancer (IBC) is a rare form of breast cancer that is highly aggressive and is associated with a poor prognosis. The disease rapidly metastasizes, and more and larger clusters of cancer cells are found both in the circulation and in the lymph vascular systems in IBC patients as compared to other breast cancer (non-IBC) patients. We hypothesized that the formation of these clusters plays a pivotal role in IBC metastasis and its rapid progression, and that their structure and function may be key to identifying molecular differences between IBC and non IBC. To date, little is known about the differences between the ultrastructure of IBC and non-IBC clusters. **Methods:** Mechanical methods were used to generate clusters of IBC cells (SUM149 and IBC3), non-IBC cells (MDA-MB-231, MDA-MB-468, and MCF7), and human mammary epithelial cells (MCF10A) by adding 2.25% of PEG8000 to the media in order to mimic the lymph fluid viscosity. Clusters were made in bacterial dishes by shaking at 40 rpm for 72 hours using a belly button dancer shaker in the CO2 incubator, then analyzed by light microscopy or prepared for and observed by transmission electron microcopy (TEM). **Results:** Significant differences were seen between IBC and non-IBC cell clusters, both by light microscopy and TEM. Light microscopy analyses revealed that IBC cells form more compact clusters than non-IBC cells. The TEM analysis revealed that IBC cells harbor numerous microvilli and microvesicles, both on the free, outer surface of the cluster as well as on the surface of cells inside the cluster. Microvilli from IBC cell clusters were both longer than those of non-IBC cell clusters and at a higher density. Dead cells were observed in the non-IBC clusters but not in IBC clusters. In addition, SUM149 cells had multiple lipid droplets in the cytoplasm. IBC3, MDA-MB-231, MCF7 and MCF10A had a few lipid droplets but not nearly as many as SUM149 cells. **Conclusions:** Cell clusters formed by both IBC cell lines, SUM149 and IBC3, exhibited a distinct ultrastructural disposition characterized by the presence of long, crowded microvilli, which was not seen in cell clusters of any other non-IBC and normal mammary epithelial cells. These microvilli may play an important role in IBC's aggressiveness compared to non-IBCs. To our knowledge, this is the first study that demonstrates the morphological differences between IBC and non-IBC cell clusters.

P312/B319

Toll Like Receptor 4/Nuclear Factor Kappa B Signaling Pathway Activation Promotes the Migration of Triple Negative Breast Cancer Cells: Inducible Nitric Oxide Synthase Role.

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Toll-like receptors (TLRs) are members of the interleukin-1 receptor (IL-1R) superfamily, play a crucial role in the inflammation and innate host defense against invading microorganisms. Toll-like receptors (TLRs) have garnered an extraordinary amount of interest in cancer research due to their role in tumor progression, invasion, survival, and metastasis. Moreover, proinflammatory pathways include several

genes that promote tumorigenesis by inducing the production of inflammatory mediators as inducible nitric oxide synthase (iNOS) and nitric oxide (NO). Accumulating evidence suggests that iNOS is involved in multiple aspects of breast cancer, including proliferation, migration, invasion, angiogenesis and response to chemotherapy. However, little research has investigated the role TLR4 activation on the migratory capacity of triple negative breast cancer cells. The aim of this research was to investigate role of TLR-4/NF-κB/iNOS signaling pathway in triple negative breast cancer cell migration. In this study, experimental evidence suggest that TLR-4 activation promotes migratory capacity of MDA-MB-231 cells, through microenvironment pro-inflammatory stimulus iNOS. TLR-4 activation by LPS, up-regulated migration (fold change ~1.5) by transwell assay and wound healing assay, through NF-κB/iNOS-dependent mechanism. Sodium Selenite (Na_2SeO_3), a NF-κB inhibitor, prevent LPS-stimulate migration and reduces expression of iNOS. LPS-stimulated MDA-MB 231 cells migration capacity is reduced in the presence of 1400W, selective iNOS inhibitor. These findings indicated a pro-migratory function that TLR4/NF-κB/iNOS signaling pathway, activated by LPS, in human breast cancer and provide a potential therapeutic target.

P313/B320

Interleukin-22 Involves in Bone Metastasis of Breast Cancer by Inducing Sphingosine-1-phosphate in Mesenchymal Stem Cell.

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Interleukin-22 (IL-22) signaling pathway has been well established to be involved in various cancer cell progressions, however, the role of IL-22 in bone metastatic breast cancer. Here, we found that bone metastatic breast cancer displays elevated expression of interleukin-22 receptor 1 (IL-22R1) and sphingosine-1-phosphate receptor 1 (S1PR1) from the GEO profiling. IL-22 stimuli promoted the expression of IL-22R1 and S1PR1 in aggressive MDA-MB-231 breast cancer cells. IL-22 treatment increased sphingosine-1-phosphate (S1P) production in mesenchymal stem cells and that induced S1P-mediated chemotactic migration of MDA-MB-231 cells. This effect was inhibited by S1PR1 antagonist. These results indicate the potential role of IL-22 in bone microenvironmtent to drive bone metastasis of breast cancer, suggesting that IL22R1-S1PR1 axis can be a potential biomarker and diagnosis in bone metastatic breast cancer.

P314/B321

Reduction of Complex Type N-glycans Suppress Neuroblastoma Cellular Properties.

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Tumor development and progression are accompanied by changes in N-glycans attached to proteins. Many secreted and membrane proteins undergo N-glycosylation, and thereby modifications in glycan processing can affect cell signaling events. Here we investigated the role of N-acetylglucosaminyltransferase-II (GnTII, *Mgat2*) protein substrates in aberrant neuronal properties of neuroblastoma (NB) cells. *Mgat2* was silenced in a human NB cell line (HuNB) to generate a novel cell line, HuNB(-*Mgat2*), which lacked complex type N-glycans, as in a rat NB cell line. Changes in the types of N-glycans were confirmed by lectin binding assays in the HuNB and HuNB(-Mgat2) cell lines, along with the rescued cell line, HuNB(-/+*Mgat2*). Further western blotting of the various cell lines

heterologously expressing a voltage-gated K+ channel (Kv3.1b) showed that N-glycans of Kv3.1b could be processed to complex type in the rescued cell line. In comparing HuNB and HuNB(-*Mgat2*) cell lines, we showed that the presence of complex type N-glycans enhanced anchorage-independent cell growth, cell proliferation and cell invasiveness, while they diminished cell-cell interactions as observed in the rat NB cell line. Cell proliferation, invasiveness and adhesion of the rescued cell lines were more like the parental cell lines than the glycosylation mutant cell lines. Further Western blotting revealed increased protein levels of MMP-2, EGFR, and Gab2 in both parental NB cell lines relative to the glycosylation mutant cell lines, and also, gelatin zymography studies demonstrated that the higher MMP-2 protein levels correlated with higher MMP-2 activity. Thus, our results support the necessity of complex type N-glycans in the promotion of cell proliferation and cell invasiveness in NB via the remodeling of the ECM and EGFR signaling pathway.

P315/B322

SNX27-retromer Assembly Recycles MT1-MMP to Invadopodia and Promotes Breast Cancer Metastasis.

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Tumor metastasis involves a complex sequence of events that facilitate the movement of the tumor cells by breaking the underlying extracellular matrix (ECM). It is composed of macromolecules mainly proteins that are densely packed like mesh-fibers that act as a physical barrier for the cellular movement. To move across the ECM, specialized actin-rich membrane protrusive structures named invadopodia are formed by the metastatic cells. Proteases that can degrade components of ECM are recycled from intracellular compartments to invadopodia for efficient degradation. This research work was aimed to study the molecular machinery governing the recycling of the proteases and thus facilitate cancer cell invasion. In the metastatic breast cancer cell line, MDA-MB-231, we found that retromer regulates the matrix invasion activity in association with sorting nexin 27 by recycling matrix metalloprotease, MT1-MMP on the cell surface. Also, MT2-MMP, another most abundantly expressed MMP, was found to be invadopodia associated in this cell line and contributes to its invasive potential. Although MT1 and MT2-MMP showed a high degree of colocalization, they were located on the distinct endosomal domains when analyzed by super-resolution microscopy. TIRF microscope-based analysis revealed that retromer and SNX27 could selectively recycle MT1-MMP but not MT2-MMP. They phenocopied each other in facilitating matrix degradation and associating with MT1-MMP. Furthermore, the in *vitro* interaction studies via pull-down and Isothermal Titration Calorimetry revealed that both SNX27 and retromer could directly interact with MT1-MMP. However, in the in *silico* analysis, among retromer and its family members, SNX27 was found to be overexpressed or profoundly altered in the patients having invasive breast cancer. In Xenograft based studies, SCID mice engrafted with SNX27 knockout cell line showed prolonged survival, suggesting possible implication for over-expression of the sorting nexin in tumor samples. These findings highlight a novel role of sorting machinery in the breast cancer cell invasion via mediating trafficking of the proteases.

P316/B323

Invasive Growth Response to DNA Repair Stress.

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Saccharomyces cerevisiae exhibits yeast form growth on rich media but some strains of *S. cerevisiae* switch to invasive growth when nutrient deprived. Our goal is to investigate the mechanism that causes invasion in *S. cerevisiae.* Specifically, we focus on DNA repair response pathways that intersect with invasive growth response pathways, based on our previous results in breast cancer cell invasiveness. Previously, we showed that transient and short-term downregulation of the expression of the DNA repair genes MRE11, RAD50, XRCC3, or RAD51 enhanced cell invasiveness in human breast cells. Here, we ask whether genetic or environmental factors that enhance or reduce DNA damage repair responses alter filamentous or invasive growth in yeast. We find that inducing DNA repair stress by a heterozygous deletion of RAD50 in an invasive strain of yeast enhances invasiveness, while challenging an MRE11 heterozygote that is non-invasive with caffeine-induced damage renders it invasive. Furthermore, mutant yeast strains with enhanced resistance to death by caffeine-induced damage display increased adhesion. Heterozygous deletion of RAD51 in invasive yeast renders it non-invasive when treated with resveratrol known to reduce oxidative damage in cells, while a significant reduction in RAD51 expression is observed in the heterozygous delete mutants of the flocculation gene FLO8 which is required for filamentous growth in diploid yeast. These results support the hypothesis that cells can be induced to become more invasive by providing a threshold level of damage to induce stress pathways relevant for filamentous and invasive growth, and provide candidate genes involved in this process.

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Loss of Awp1 Leads to Enhanced Cell Motility of Breast Cancer Cells.

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Metastatic breast cancer is leading cause of cancer-associated deaths in women worldwide, however, the prognosis for detection of early stage breast cancer go on to develop metastatic disease is still lacking. Therefore, targeting tumor cell motility is one of strategies to improve the outcome for poor prognosis in patients with breast cancer. Here, we found that AWP1 (ZFAND6, zinc finger AN1 type-6) expression level is lower in the aggressive breast cancer cells compared with non-aggressive breast cancer cells. AWP1 knockdown using short hairpin RNA in non-aggressive MCF7 cells promoted cell motility. Furthermore, CRISPR/Cas9-mediated AWP1 knockout led to morphological changes towards a mesenchymal cell type and also accelerated the invasion of MCF7 cells. Our findings suggest that loss-of-function of AWP1 promotes the migration capacity of breast cancer cells, linking to the cancer invasion and further formation of metastasis.

P318/B325

Phenotypical and Signaling Pathway Alterations in Fibroid Cells Following Continuous Cadmium Exposure and the Implications in Carcinogenesis.

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Background: the heavy metal Cadmium (Cd), a widespread environmental contaminant, poses serious hazards to human health. We found that Cd can act as a metalloestrogen, stimulating growth of hormonally responsive human uterine leiomyoma (ht-UtLM; fibroid) cells. In women of reproductive age, a statistically significant association between blood Cd levels and fibroid volume has been observed. Prolonged exposure to Cd and Cd-containing substances, such as cigarette smoke, is thought to contribute to cancer progression. **Objective:** the aim of this study was to determine if benign fibroid cells could be altered in *vitro* by continuous Cd exposure. **Experimental Design:** Fibroid cells were exposed to 10 µM $CdCl_2$ for 8-weeks to evaluate the impact of Cd exposure. **Methods: the** Cd exposed cells were analyzed by time-lapse confocal imaging, flow cytometry, transmission electron microscopy, Ki-67, and soft agar assays. Total RNA was extracted and analyzed using NanoString PanCancer Progression/Pathways Panels. **Results:** When fibroid cells were exposed to 10 µM $CdCl_2$ for 8 weeks, a robust and fast-growing Cd Resistant LeioMyoma culture (abbreviated as CR-LM) was established. CR-LM cells contained increased cytoplasmic aggregates of glycogen at the cell periphery and had significantly enhanced cell motility compared to passage-matched controls. A higher percentage of CR-LM cells were in the G2/M phase of the cell cycle and had dramatically increased Ki-67 (a proliferation marker) labeling indices. CR-LM cells were able to form viable colonies in soft agar thus exhibiting anchorage-independent growth. NanoString analysis showed the downregulation of genes encoding for extracellular matrix (ECM) components, such as collagens (COL1A1, COL3A1, COL4A1, COL5A1), fibronectin, laminins (LAMA3, LAMA4), and SLRP family proteins (LUM, BGN, DCN and FMOD); whereas genes involved in ECM degradation were dramatically upregulated (MMP1, MMP3 and MMP10). A protein antibody array technique was used to corroborate the NanoString MMP expression data. Ingenuity Pathway an alysis showed that TGF-Beta signaling network was significantly suppressed due to the Cd exposure, and the inhibited TGF-Beta signaling network have statistically strong associations with a heightened cell proliferation state. **Conclusion:** Due to prolonged Cd exposure, fibroid cells developed altered phenotypes in cell morphology, motility, proliferation, glycogen distribution, anchorage independent growth, and pathway signaling. **Impact statement:** Continuous chronic environmental Cd exposures may pose a cancer risk for women with uterine fibroids.

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Cancer Therapy 1: Novel Therapies and Drug Delivery

P319/B326

Novel Combination Treatment Designed to Target Both Metastatic Cells and Proliferation in Pancreatic Ductal Adenocarcinoma.

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Pancreatic cancer is the 4th most common cause of cancer-related deaths in the United States with only 7-9% 5-year survival rate. While there are a few combination treatments that have been shown to increase overall survival by several months, gemcitabine monotherapy, which has been used to treat

PDAC since 2006, is still the most commonly administered first-line treatment. Our objective is to improve patient survival by providing a novel combination therapy designed to inhibit the growth of solid tumors and prevent metastatic cancer cells from reaching secondary organs. Our lab has recently shown that secreted interleukin 6 (IL-6) and interleukin 8 (IL-8) can induce a migratory phenotype in tumorigenic, metastatic cancer cells in triple negative breast cancer models. Cells exposed to these cytokines display enhanced invasion through stromal environments, and this phenotype can be reversed by blocking the IL-6 and IL-8 receptors with the combined treatment of tocilizumab and reparixin. We hypothesized that this simultaneous blocking of IL-6 and IL-8 receptors would improve PDAC patient outcomes when used in combination with gemcitabine by targeting both proliferation and metastasis. Preliminary results show that this combination treatment has a positive effect in in vivo PDAC models. Our novel treatment combination was able to significantly reduce the tumor size, exceeding that seen from gemcitabine monotherapy. In addition, the triple combination reduced the metastatic burden and normalized the effect of the treatment among the mice in the group.

P320/B327

Identification of a Novel Compound That Targets Hepatocellular Carcinoma.

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The death rates for hepatocellular carcinoma (HCC) are rising faster than the death rates of any other cancer in the United States. Our group has identified a unique intranuclear pathway for nuclear export of a subset of viral mRNAs and found that constituents of this pathway also regulate nuclear export of a subset of cellular mRNAs that encode regulators of tumor metastasis. Based on a high-throughput screen of 232,500 chemical compounds from our unique compound library, we have identified 22 small molecules that specifically inhibit this RNA export pathway without compromising bulk cellular mRNAs. One of the 22 compounds preferentially kills HCC cells with minimal toxic effects on normal liver cells. Our study shows that this compound reduces HCC cell growth, invasion and colony formation. In the future, we propose to test this compound in various mouse models, including human and patient derived xenografts introduced into immune suppressed mice. Finally, we will determine the genetic alterations that confer sensitivity or resistance to our compounds. Taken together, our studies may lead to the identification of a novel compound that specifically targets HCC, a cancer type that currently has no targeted therapies.

P321/B328

Investigating the Anti-proliferative Effects of a Derivative of Bitter Melon, Tubeimoside I, on Cancer Cells.

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2019 Research Abstract Rachel Villareal **Investigating the Anti-Proliferative Effects of a Derivative of Bitter Melon, Tubeimoside I, on Cancer Cells** Although there have been advancements in treatments towards cancer, scientists have not yet found a cure. Current studies suggest some natural supplements can serve as alternative treatments for cancer, with the benefit of reduced to no side effects. *Momordica charantia* Linn, also known as Bitter melon, is a popular Asian herb used in traditional medicine to treat cholera, anemia, blood diseases, liver and spleen disorders, and diabetes. While, Bitter melon contains various active ingredients (terpenoids, sterols and fatty acids), Tubeimoside I (TBMS1) has been identified as exhibiting antitumor properties in two blood cancers, leukemia and lymphoma,

through the induction of cell cycle arrest and death. Our previous study investigated the cell viability, cytotoxicity, and caspase mediated apoptosis when the cancer cells were exposed to TBMS1. These results showed that both liquid (non-adherent cancer cells) and tumor forming (adherent cancer cells) cancer cell lines experienced a significant decrease in cell viability and increase in cytotoxicity when exposed to a minimum of 20 uM TBMS1. Therefore, this study explored the mechanism through which TBMS1 exerts its effects on these cancer cell lines. Possible mechanisms of death would be apoptosis, anoikis, autophagy, or necrosis for breast and prostate cancer cell lines. Briefly, MCF7, LNCap, and Jurkat cell lines were exposed to a 25 uM concentration of TBMS1 over a period of 48 hours and assessed for Necrosis and Apoptosis mechanisms. This analysis shows that the MCF7 cell line experienced apoptosis first and then secondary necrosis while the LNCap and Jurkat cell lines experienced necrosis as the primary manner of cell death.

P322/B329

Investigation of Novel Small Molecule Ferroptotic Compounds.

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Ferroptosis is a form of iron-dependent cell death characterized by elevated lipid peroxides and reactive oxygen species (ROS). We have identified a novel class of small molecules that induce ferroptosis in certain types of cancer cells. ROS is produced as a byproduct of metabolism in almost every cell. Glutathione (GSH) plays an essential role in scavenging ROS to maintain cell viability, and acts as a cofactor of Glutathione peroxidase 4 (GPX4) that protects lipids from oxidation. Cysteine, provided by uptake of extracellular Cystine, is essential for GSH synthesis. Ferroptosis occurs via two mechanisms: blocking the Cystine importing system Xc^- (Type I inducer) or inhibiting GPX4 activity (Type II inducer). System Xc^- is an antiporter transmembrane protein which imports Cystine and exports Glutamate. Our previous work indicated that our compound 6E decreased glutamate secretion as well as reduced thiols (measured using monochlorobimane; MCB) consistent with a type I mechanism. Here we show using FITC-conjugated cystine, that indeed, cystine import is reduced by 6E. We also investigate the specificity of our previous result with MCB. Since GSH is the most abundant reduced thiol in many mammalian cells, MCB is widely used as a marker of GSH. However, other thiols may contribute to results with this dye. Therefore, we transfected MDA-MB 231 cells with the biosensor Grx1-roGFP2 to measure the level of intracellular oxidized glutathione (GSSG). Grx1 uses glutathione to reduce roGFP2 altering its spectral properties. Proximity of Grx1 to roGFP2 provides a measure of glutathione in live cells. Upon treatment with 6E, we found a higher level of GSSG confirming our previous results with MCB. All these results together are consistent with our hypothesis that 6E kills via Type I mechanism. Even though ferroptosis is a well-established form of cell death, signaling pathways that modulate this process are not known. Therefore, we used a panel of growth factors/kinase inhibitors to test effects on 6E induced ferroptosis. We discovered that two chemically distinct inhibitors of IGF1 and insulin receptors could rescue from 6E mediated cell death. However, addition of excess IGF1 did not sensitize cells to ferroptosis. Either insulin signaling is maximal in our cell lines or our tyrosine kinase inhibitors block ferroptosis via alternative mechanisms. Ongoing research will address these questions.

P323/B330

3D Bioscaffolds from Bovine Bone Marrow: a Model for Testing New Drugs Targeting Hematopoietic Malignancies.

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Myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) are hematopoietic malignant disorders with low chances of response to chemotherapy and high mortality rates. Furthermore, chemotherapy treatment adds considerable morbidity to the patients who are, frequently, older than 60 years. Thereby, advances in the understanding of molecular targets associated with their pathogenesis as well as the development of new drugs and testing strategies have been the subject of research over the past years. We have previously identified an overexpressed gene, *hematopoietic cell kinase* (HCK), in bone marrow hematopoietic stem cells (HSC) from MDS and AML patients, which activates the PI3K and MAPK pathways, increases cell growth and reduces apoptosis. We then produced a chemical inhibitor (iHCK), which showed selective efficacy for leukemic cells and did not affect normal HSC (Roversi *et al.* BBA Mol Basis Dis. 2017, 1863(2):450-61). In order to study the activity of this new pharmacological compound in MDS and AML cells in a more physiological environment, we developed a 3D bioscaffold by decellularization of bovine bone marrow that enables co-cultures and mimics the bone marrow niche (Bianco *et al.* Biomat Sci 2019, 7(4):1516-28). After informed written consent and approval of the Ethical Committee of University of Campinas (CAAE 1000.0.146.00-11), in accordance to the Helsinki Declaration, HSC were isolated from bone marrows of healthy donors (HD), MDS and AML patients and were treated or not with iHCK in liquid culture containing X-Vivo medium plus cytokines (SCF, IL-3, IL-6, TPO) for three days. Meanwhile, HS-5 mesenchymal cells were stirred for 3h before being cultured into 3D bioscaffolds with DMEM and fetal bovine serum. iHCK or vehicle (DMSO) treated HSC were introduced into 3D bioscaffold containing or not HS-5. 3D bioscaffold was evaluated after 7 and 14 days, by light microscopy (hematoxilin and eosin regular staining) and immunohistochemistry (expression of CD34 and CD90 antigens). Results showed that iHCK treatment reduced MDS and AML hematopoietic stem cells proliferation when compared to HD HSC in the 3D bioscaffold containing HS-5 cells and confirmed the crosstalk between hematopoietic and mesenchymal cells since normal or malignant HSC were preferentially located in areas containing HS-5 cells. Indeed, HSC cells have a very low growth in 3D bioscaffolds without HS-5 cells. In conclusion, selective HCK inhibition is a promising strategy for MDS and AML treatment and 3D bioscaffolds obtained from bovine bone marrow represent a potential platform for hematopoietic stem cells expansion and testing of new drugs.

P324/B331

Anti-proliferative and Apoptotic Inducing Activity of Novel *2-deoxy-d-glucobenzotriazoles* on Cancer Cells.

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A hallmark of cancer cells is characterized by an increase in glucose consumption and aerobic glycolysis. Cancer cells are known to rewire their metabolism to promote unregulated tumor proliferation and survival and meet high demands for energy. The alteration of metabolic pathways has become a target for developing strategies that exclusively affect cancer cells and not normal cells. In this study, we were

interested in developing chemical inhibitors that target glycolysis in cancer cells but minimally inhibit normal cell growth. Based on previous findings on the biological effects of benzotriazoles, a series of novel benzyl, acetyl and fully deprotected glycosylbenzotriazoles were chemically synthesized and screened for their anti-proliferative properties. Modifications were made to these agents based on the findings of our study and the analysis of various protecting groups and lipophilic nature of the compounds tested. Using HeLa cells, a cervical cancer cell line, we performed MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) cell viability assays 24 hours post-treatment at various concentrations of these compounds. We found that compounds OBn-162 and OAc-220 significantly decreased cell viability compared to DMSO-treated control cells. We next wanted to test the most effective compounds using a primary cell line. We used Human M2 macrophages that have been stimulated using M-CSF (macrophage colony stimulating factor) and found that the compounds tested may have a reduced cytotoxic effect on normal cells compared to HeLa cells. Finally, we found that cell viability and apoptosis synergistically increased when cells were treated with both OBn-162 and the apoptosis-inducing chemotherapeutic drug Paclitaxel simultaneously. Taken together, our studies provide evidence for potential use of these compounds and their derivatives as chemotherapeutic agents. However, more work must be done to determine the exact mechanism of these compounds and to determine their effects on other cancer and normal cell lines.

P325/B332

Characterization of a New Microtubule-depolymerizing Compound with Anti-cancer Properties.

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We synthesized a new family of compounds with anti-cancer properties. Microtubule-targeting agents are some of the most successful chemotherapies that are still in use. However, they can also affect healthy cells causing serious side effects, and patients often develop resistance to these drugs. Thus, there is a desire to increase the repertoire of anti-cancer drugs. We strategically designed a scaffold with several functional groups amenable to modifications for structure-activity-relationship studies. Importantly, the compounds were designed with properties ideal for in *vivo* use, and to probe molecular space, but without a specific target in mind. Of ~40 derivatives, we found that several have high efficacy in a myriad of cancer cells, while others have no or little effect, suggesting that efficacy can be attributed to changes in the functional groups and is not due to the scaffold. Our lead compound, C75, has IC_{50} for viability ranging from 100-200 nM in breast cancer cells, 300-400 nM in cervical, lung, colorectal cancer cells, 800 nM in fibroblasts in culture, and >1 uM in healthy fibroblasts from patients. We found similar efficacy in multi-cellular tumor spheroids (MCTS) derived from some of these cell lines. To determine C75's mechanism of action, we performed studies in cells and in *vitro*. Flow cytometry and imaging studies revealed that C75 arrests cells in mitosis. Staining cells for gamma-tubulin, centrin-2 and tubulin revealed that C75 preferentially affects spindle poles, which fragment forming multi-polar or disorganized spindles. To determine if C75 directly binds to tubulin, we performed in *vitro* binding and polymerization assays. C75 effectively blocked polymerization at concentrations lower than colchicine, a well-characterized compound known to disrupt polymerization. Competition assays revealed that C75 was able to compete with colchicine for tubulin-binding, similar to nocodazole. However, this competition was not sustained over time, suggesting that although C75 binding to tubulin could overlap with the colchicine site, it does so less efficiently. In addition, the spindle phenotypes caused by C75 in cells are different compared to colchicine, and they show synergistic phenotypes when combined. This data suggests that C75 has unique accessibility to microtubules, preferentially binds to a different site on

tubulin, and/or has an additional molecular target in cells. Thus, we have identified a new, modifiable tubulin-targeting compound with high selectivity towards breast cancer that warrants additional in *vivo* studies.

P326/B333

Sensitization of Multidrug Resistant Cancer Cells HCT-116 by Surface Modified PAMAM Dendrimer with Gallic Acid.

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Cancer treatment is restricted due to the resistance development in cancer cells by chemotherapeutic drugs. Resistance to chemotherapy is massive challenge in cancer therapeutics. Therefore, it is important to address this issue. Gallic acid (GA) is a natural plant compound that exhibits various biological properties including anti-proliferative, anti-inflammatory, anti-oxidant and anti-bacterial. Despite of the wide spectrum biological properties GA has cytotoxic response, low solubility and low bioavailability. To overcome this problem, GA was conjugated with the Polyamidoamine (PAMAM) dendrimer for improving the bioavailability and efficient delivery in drug resistant HCT-116 Colon Cancer cells. In our study we have modified surface of PAMAM dendrimer with Gallic acid and examine their anti-proliferative effects. Further, drug resistant colon cancer cells were established and thereafter treated with different concentration of PAMAM-GA to examine their anti-proliferative potential and underlying mechanisms of multidrug resistance in cancer cells. Our results show that PAMAM-GA conjugate induces apoptotic cell death in HCT-116 and drug-resistant cells examined by an nexin-PI staining and western blot of caspase-9 and caspase-3. In addition, it also shows that multidrug resistant drug transporter P-gp protein expression was down regulated with increasing the concentration of PAMAM-GA conjugate. After that we also observed the significant difference in Rh123 efflux and accumulation in drug sensitive and drug resistant cancer cells. Thus, our study suggests that conjugation of anti-cancer agents with PAMAM could improve drug resistant property and cytotoxic response to treatment of cancer. Keywords- P-gp, Drug resistance, Gallic acid, PAMAM dendrimer, Apoptosis

P327/B334

Modulating Ruthenium Induced Trafficking of Atp7b to Overcome Drug-resistance in Cancer.

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The copper ATPase, ATP7B resides in Trans-Golgi Network (TGN) and provides copper to the secretory pathway. When copper level rises in cytosol ATP7B vesicularizes and traffics to the plasma membrane (PM) to excrete out excess copper. ATP7B has 6 Metal Binding Domains (MBDs) in cytosolic N-terminal that works as copper sensors. Due to its Cys residue (CXXC) in the MBDs, ATP7B also sequesters Platinum(Pt) based chemotherapeutic drugs and traffics to export it out. Ruthenium (Ru) based compounds due to their anticancer properties against resistant-cancer are potential therapeutic alternatives to Pt resistant cancers. We have investigated if Ru-complexes are also disposed to ATP7B based sequestration and export. Further, we have designed Ru-complexes that can bypass ATP7B sequestration and hence promises to serve as a better chemotherapeutic. We have developed a series of $Ru^{II}(\eta^6$-*p*-cymene) complexes that showed cytotoxicity against cancer cells. We found that unlike Cisplatin (CDDP), Ru complexes showed no change in the expression level of ATP7B, making it a

preferred choice over Pt-drugs. Trafficking of ATP7B from TGN to vesicles was used as a measure of drug export. We performed immunofluorescence in Ru compounds (**chloro**) treated HepG2 cells and found ATP7B to traffic. We confirmed the phenomena performing time-lapse fluorescence microscopy where trafficking of ATP7B was triggered in minutes of addition of the drug. But when the same was performed with Ru complex carrying a different ligand (**iodo**), we observed absence of ATP7B vesicularization from TGN. Immunostaining in HepG2 cells revealed that though vesicularization was absent, ATP7B exits TGN and shows tight perinuclear accumulation. Measurement of Ru concentration in cell revealed the iodo-Ru complex is exported less from the cell than its chloro counterpart. To establish sequestration of Ru by ATP7B, we used a previously established assay where *E.coli* form filaments upon CDDP treatment and are rescued by expressing N-Terminal of ATP7B (N-ATP7B) in the bacteria, suggesting drug sequestration. We were able to rescue *E.coli* cells from Ru toxicity by expression of N-ATP7B. But in the case of iodo-Ru we observed *E.coli* filamentation, suggesting no sequestration. Among the Ru-compounds tested, iodo-Ru shows least binding to GSH. Presently we are trying to understand the molecular mechanism of binding of Ru-complex with the purified N-terminus of ATP7B using Isothermal Titration calorimetric studies. ATP7B play a major role in resistant to Ru-based anti-cancer agents similar to CDDP. In this study, we have designed Ru-compounds that escape ATP7B sequestration and export, hence may be a better alternative to Pt drugs.

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Taxane Resistance in Breast Cancer Controlled by the Apc Tumor Suppressor.

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Adenomatous Polyposis Coli (APC) is a multi-domain tumor suppressor protein that binds to proteins including β-catenin, axin, and microtubules (MTs). APC is lost in many epithelial cancers and up to 70% of sporadic breast cancers, with a tendency towards triple negative breast cancers (TNBCs). Our laboratory previously demonstrated that APC knockdown in the human TNBC cell line, MDA-MB-157, resulted in resistance to the Taxane family chemotherapeutic agent, Paclitaxel (PTX). Given that PTX and APC both alter MT dynamics and impact the G2/M phase of the cell cycle, we sought to understand the molecular mechanisms of APC-mediated resistance. We **hypothesized** that genes involved in the G2/M transition would be altered in the absence of APC, leading to PTX resistance. We first performed an unbiased analysis of transcriptomic changes downstream of APC loss to identify potential therapeutic targets to overcome PTX resistance. In this, a cadre of transcripts involved in regulation of the cell cycle were identified, including AR, FOXS1, and GLI1. Upon validation of results by qRT-PCR and western blot, studies in the laboratory will investigate the effect of manipulating expression of these genes on the response to PTX. Upon further investigation into the cell cycle, we observed no change in the G2/M arrest induced by PTX. However, analysis of G2/M checkpoint proteins, CDK1 and cyclin B1, showed a significant increase in CDK1 expression in APC^{KD} cells compared to control. Nuclear fractionation demonstrated that nuclear CDK1 localization is increased after PTX treatment selectively in the APC shRNA cells. Based on these findings, we performed combination studies using a CDK1 inhibitor (RO-3306) with PTX treatment. PARP (total and cleaved) was used to measure apoptosis, and showed enhanced cleavage in the APC^{KD} cells specifically after combination treatment. Overall, our studies strive to understand the molecular nature of PTX resistance in APC^{KD} cells, and to identify therapeutic target(s) for APC-mutant TNBCs.

P329/B336

Decreased Expression of Eno-1 Promotes Cisplatin Resistance by Inducing Senescence and Altering Metabolic Pathways.

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Ovarian Cancer is the fifth leading cause of death among women worldwide. It accounts for 23,000 deaths in the US alone. The standard treatment for this malignancy is surgical tumor removal in combination with platinum-based chemotherapy. Despite initial good response, patients develop resistance to platinum-based drugs which results in recurrence of the disease. Several mechanisms for platinum-resistance have been proposed, however the events leading up to resistance remain elusive. Therefore, it is imperative to study the molecular pathways leading to cisplatin resistance. Molecules in these pathways will be further used as therapeutic targets for platinum resistant ovarian cancer. Aimed to identify proteins associated with cisplatin resistance we performed proteomic studies in cisplatin sensitive (A2780) and cisplatin resistant (A2780CP20) ovarian cancer cells. One of the most dysregulated proteins in these cells was Enolase-1 (ENO1), an enzyme involved in the glycolytic pathway. ENO1 was found to be reduced in cisplatin resistant cells (A2780CIS, A2780CP20, OV-90CIS, OVCAR3CIS) as compared to their sensitive counterparts (A2780, OV-90, OVCAR3). We hypothesize that a way to acquire resistance is through senescence along with a reprograming of the metabolic pathways, using glycolysis as the main source of ATP, and mitochondrial respiration as an external source of energy. First, we evaluated the role of senescence by analyzing the levels of beta-galactosidase present in an Ovarian Cancer cell panel. We found that ovarian cancer resistant cell lines exhibit high levels of beta-galactosidase as compared to their sensitive counterpart indicating the presence of senescent cells. To further validate this results, we evaluated the levels of p21 in these cells. P21 is involved in cell cycle arrest, maintaining cell viability while leaving it in a senescent state. Furthermore, we analyzed intracellular glucose in the ovarian cancer cell panel and observed that cisplatin resistant cells have an increase in glucose accumulation as compared to their sensitive counterparts. We also measured the ATP levels and our preliminary results suggests higher ATP consumption in cisplatin resistant cells compared with their sensitive. Taking this into consideration, we performed clonogenicity assays under different nutrient conditions (glucose, glutamine, galactose, and pyruvate) in cisplatin resistant cells (A2780CP20). These results will help us to understand the molecular mechanism by which populations of ovarian cancer cells become more resistant to cisplatin treatment.

P330/B337

Anti-tropomyosin Drugs Prevent the Rescue of Vincristine-induced Mitotic Spindle Defects.

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Drugs targeting a major component of the actin filaments of cancer cells, tropomyosin Tpm3.1, synergize with anti-microtubule drugs in neuroblastoma and lung cancer models both in *vitro* and in *vivo* and a wide range of other cancer types in *vitro*. We have determined the mechanism of synergy in HeLa cells to gain insight into the potential interaction of actin filaments and microtubules in the survival and proliferation of cancer cells. HeLa cells exhibit a strong synergistic response to the combined treatment

of vincristine (VCR) and anti-Tpm3.1 compounds, marked by an enhanced reduction in cell viability, apoptosis induction and mitotic cell cycle arrest. Tpm3.1 localizes to the cell cortex during mitosis, potentially associating with the microtubule network, particularly the dynein/dynactin complexes responsible for mediating cortical pulling forces during spindle assembly. VCR alone causes supernumerary NuMA organized acentrosomal microtubule organizing centers upon nuclear envelope breakdown, which can be resolved via a clustering mechanism to achieve bipolar cell division. The addition of anti-Tpm3.1 compounds inhibits NuMA-associated clustering in VCR-treated cells, leading to irreparable defects during spindle assembly and thus a largely increased number of cells with multi-polar spindles undergoing mitotic delay and catastrophe. We conclude that actin/Tpm3.1 filaments contribute to the formation of the bipolar spindle and play a critical role in the clustering of acentrosomal microtubule asters.

P331/B338

Quercetin Encapsulated Thermosensitive Liposome-gold Nanoparticles Depolymerize Microtubules, Suppress Hsp70 Expression and Induce DNA Damage in Cancer Cells After Photothermal Activation.

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The overexpression of heat shock protein 70 (Hsp70) during photothermal therapy (PTT) leads to the development of thermotolerance in cancer cells. Quercetin (QE), a bioflavonoid has several targets such as Hsp70, tubulin, and phosphatidylinositol 3 kinase. QE is known to bind to tubulin and to inhibit tubulin polymerization. We hypothesize that by combining QE along with PTT will improve the therapeutic efficacy. Herein, we report QE encapsulated biodegradable thermosensitive liposome- gold (QE-LiposAu) nanoparticles for PTT of hepatocellular carcinoma. QE-LiposAu nanoparticles were prepared by in *situ* reduction of chloroauric acid using ascorbic acid in the presence of QE encapsulated thermosensitive liposomes. QE-LiposAu nanoparticles were characterized using electron microscopy, dynamic light scattering, and zeta potential measurements to confirm the gold coating on the liposomes. The four-fold increase in QE release at 45 °C with respect to 37 °C confirmed the thermosensitivity of DSPC: Chol (75:25 molar ratio) liposomes. QE-LiposAu nanoparticles were found to have a photothermal conversion efficiency ~ 75 % which makes QE-LiposAu nanoparticles an efficient photothermal agent. QE-LiposAu nanoparticles caused apoptosis mediated cell death upon 750 nm near infra-red laser (650 mW) treatment for 5 min. Further, we observed QE-LiposAu nanoparticles mediated PTT depolymerized microtubules, suppressed Hsp70 expression and induced DNA damage in hepatocellular carcinoma. Also, QE-LiposAu nanoparticles showed hemocompatibility, indicating its potential for in *vivo* application. The results indicated that these biodegradable and thermosensitive QE-LiposAu nanoparticles can be promising photothermal agents for cancer therapy.

P332/B339

Multifunctional Nanoparticles for Genetic Engineering and Bioimaging of Natural Killer (nk) Cells Therapeutics.

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Recently, natural killer (NK)-based immunotherapy has attracted attention as a next-generation cell-based cancer treatment strategy due to its mild side effects and excellent therapeutic efficacy. Here, we describe multifunctional nanoparticles (MF-NPs) capable of genetically manipulating NK cells and tracking them in vivo through non-invasive magnetic resonance (MR) and fluorescence optical imaging.

The MF-NPs were synthesized with a core-shell structure by conjugation of a cationic polymer labeled with a near-infrared (NIR) fluorescent molecule, with the aid of a polydopamine (PDA) coating layer. When administered to NKs, the MF-NPs exhibited excellent cytocompatibility, efficiently delivered genetic materials into the immune cells, and induced target protein expression. In particular, the MF-NPs could induce the expression of EGFR targeting chimeric antigen receptors (EGFR-CARs) on the NK cell surface, which improved the cells' anti-cancer cytotoxic effect both in vitro and in vivo. Finally, when NK cells labeled with MF-NPs were injected into live mice, MF-NP-labeled NK cells could be successfully imaged using fluorescence and MR imaging devices. Our findings indicate that MF-NPs have great potential for application of NK cells, as well as other types of cell therapies involving genetic engineering and in vivomonitoring of cell trafficking.

P333/B340

Functionalization of Gold Nanoparticles by the *Clostridium Perfringens* Enterotoxin C-terminus *for* an Optical Induced Ablation of Tumor Cells.

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Expression of claudins is increased in tumor cells. We proposed to conjugate the C-terminus of the *Clostridium perfringens* enterotoxin (C-CPE) to gold nanoparticles (AuNPs) to produce a C-CPE-AuNP complexes (C-CPE-AuNPs). By binding to claudins in cell membrane, the C-CPE moiety should allow to put the AuNPs in close vicinity of cells, allowing a subsequent specific cell killing by laser induced optoperforation. The human Caco-2, MCF-7 and OE-33 as well as the canine TiHoDMglCarc1305 were identified as tumor cells expressing claudin-3, -4 and -7. Functional analysis showed that the cells formed good transepithelial barrier which was characterized by a high electrical resistance (TEER) and was perturbed by application of recombinant produced C-CPE. The recombinant C-CPE was used to generate C-CPE-AuNPs, which allowed to specifically eliminate the cells expressing claudins using the gold nanoparticle mediated laser perforation (GNOME-LP) technique. The efficiency of cell killing was related to the C-CPE concentration used for the AuNP functionalization. The scanning velocity was varied from 5 mm/s to 40 mm/s and the laser fluence was varied between 0 mJ/cm^2 and 75 mJ/cm^2. The maximal efficiency (> 90%) was achieved when the AuNPs were functionalized using 20 µg/ml C-CPE, scanned at 5 mm/s using a laser fluence of 20-30 mJ/cm^2. In 3D matrigel culture system, the optical treatment in presence of C-CPE-AuNPs completely destroyed spheroid composed of Caco-2 cells and reduced OE-33 cells spheroid formation. At cellular level, the optical treatment of the cells that bound C-CPE-AuNPs were stained by annexin V and showed reduced mitochondria activity. However, an enhancement of caspase 3/7 activity in the cells was not found and DNA analysis did not show apoptosis related DNA ladder. The results gives the physical parameters for an optimal AuNP functionalization using C-CPE as well as optoperforation. Moreover the results suggest that the combination of C-CPE-AuNPs and optical treatment induced a massive cell permeabilization followed by necrosis related cell death.

P334/B341

Antibody Conjugated Nanoparticles Solid Lipid Nanoparticles as Drug Delivery Systems for Hydrophobic Pharmaceuticals in Cancer Therapy: Synthesis, Uptake, and Effect.

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Despise the progress in prevention, detection, and treatment of cancer, the disease remains at the second place in causes of death in the world. Currently, one of the main treatment methods includes the use of chemotherapeutic agents such as small molecule drugs. However, these small molecule drugs give rise to challenge that includes poor solubility, bioavailability, and absorption within the body and the destruction of normal cell tissue, toxicity, and the risk of development of drug resistance. In the last two decades, there has been an increased interest in nanoparticles both in terms of diagnostics and drug delivery for various diseases including cancer. The focus of this study is the development of a targeted drug delivery system for cancer therapy composed of solid lipid nanoparticles (SLNs). Antibody conjugated SLNs in the sub-500 nm range are synthesized for use as an actively targeted drug delivery system using a simple and inexpensive one-step synthesis of SLNs capable of encapsulating small molecule drugs. The SLNs consists of a lipid core which surface is conjugated to both an albumin stealth corona and targeting antibodies (anti-EGFR) and are designed to release the cargo by disassociating when entering the intracellular compartment. Using this one-step synthesis method, SLNs with diameter down to 89 nm and zeta potential of -29 mV was obtained. Cellular uptake of the SLNs loaded with the model drug Nile Red was examined in *vitro* in CAL27, HeLa, hMSC, and H1299, respectively. It was found that albumin coating stabilized the SLNs against aggregation in serum and reduced non-specific cell uptake. Furthermore, antibody conjugation of the stealth SLNs led to a marked cellular retargeting thus increasing the uptake of the model drug. Additionally, free-floating SLNs binding to the cell surface for subsequently being internalized within the time range of ~30 minutes to 2 hours have been visualized. Finally, the SLNs were tracked within the cells to determine their fate by observing colocalization with early and late endosomes as well as lysosomes where it was found that the SLNs are capable of endosomal escape and avoid lysosomal degradation. This SLNs system is simple to synthesize, have good serum stability and stealth properties and have the potential to be versatile targeted towards cancer cells using antibodies.

P335/B342

Copper Sulfide and Folate Nanoparticles and Photothermal Therapy: Novel Therapy for Gynecological Cancers?

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The gynecological cancers, endometrial, ovarian and cervical cancer, affect over 30% of women nation-wide, with a combined mortality rate of over 10%. Endometrial cancer is the most common gynecological cancer, but ovarian cancer is the deadliest. The incidence of cervical cancer has decreased significantly over the years due to Pap tests, but its mortality rates are still too high, if the disease is not detected in the early stages. While treatments of surgery, chemotherapy and radiotherapy have been successfully used to treat gynecological cancers, there is still a relatively high incidence of relapse. Hence, the development of novel therapeutic approaches such as, photothermal therapy (PTT) used in combination with nanoparticles, that would decrease the incidence of relapse and detrimental treatment side effects, is of utmost importance. Recent studies have indicated that the increase in

temperature during PTT could kill targeted cancer cells, but normal cells could also be damaged by heat conduction. It has also been determined that if a cell is targeted by engulfing a nanoparticle, such as Folate/Folic acid (FA), then cancer cells that express FA receptors will absorb FA and be greatly affected during PTT. Therefore, the objective of this project was to determine the effect(s) of PTT, using copper sulfide (CuS) and folic acid (FA) nanoparticles, on endometrial (RL95-2); ovarian (SKOV-3) and cervical (HeLa) cancer cell lines. We hypothesized that the aforementioned cells treated with a CuS-FA nanocomposite, then subjected to PTT will be less viable than control cells. To test this hypothesis, cultured RL95-2, SKOV-3 and HeLa cells, and A549 cells (a lung cancer cell line that is FA receptor-negative) were treated with the following combination of nanoparticles: (1) 250ng of CuS; (2) 250ng FA; (3) 250ng of CuS-FA nanocomposites and (4) 0ng (control); for a period of 48 hours. All cells were then exposed to PTT at the near infrared region (900 nm wavelength) for 10 minutes, then subjected to an MTT assay to determine cell viability. Relative to control (0ng) cells, there was a significant decrease in viability in the RL95-2, SKOV-3 and HeLa cells treated with the CuS-FA nanocomposite and PTT. The decrease in viability was highest in HeLa cells, followed by SKOV-3 cells then RL95-2 cells, then A549 cells. This observation could be attributed to the presence of FA receptors being highest on HeLa cells, followed by SKOV-3 cells. Further studies of PTT/CuS nanoparticles and their effects on the TGF-β gene and gene products in the RL95-2, SKOV-3 and HeLa cells, would provide data that could improve therapy for gynecologic cancers.

P336/B343

Pluronic Micelles Encapsulated Curcumin Potentiates Apoptosis and Anti-inflammatory Response in Drug Resistant Human Breast Adenocarcinoma Cells.

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Chemotherapy is the most common treatment for cancer. But after some time cancer cells acquire resistance against chemotherapeutic drugs. Therefore, multidrug resistance is one of the serious concerns in the failure of anti-cancer therapies and alternative therapy is the need of the hour. Natural flavonoids may be the better alternatives of chemotherapeutic agents since few decades but poor bioavailability of many drug candidates is one of their limitations. Curcumin is the natural polyphenolic compound derived from rhizome of the plant *Curcuma longa* which is a well known chemopreventive and therapeutic agent. However, the pharmacological application of curcumin is obstructed due to low bioavailibility and rapid systemic elimination. In the present study, we have synthesized aqueous soluble Pluronic Micelles encapsulated Curcumin (PMsCur) to combat drug resistance in cancer cells. Pluronics are biological response modifiers and display an ability to interact with hydrophobic surfaces and biological membranes. In this regards, we have established drug resistant human breast adenocarcinoma cells (MCF-7/R) and analyzed biological activities of PMsCur. Our studies revealed that PMsCur downregulates P-glycoprotein which is a drug efflux transporter linked with multiple drug resistance and CD44v6 which is a cancer stem cell marker. Cytotoxicity studies suggest that PMsCur efficiently inhibits the proliferation of MCF-7/R cells as compared to free curcumin. Further, we explored the mechanism of cell death and found that PMsCur exhibits apoptosis by mitochondrial disruption and activation of effector caspases in MCF-7/R cells. Moreover, the inflammatory study revealed that PMsCur inhibits NF-kB activity and downregulates proinflammatory cytokines. Interestingly, PMsCur modulates the expression of redox regulating enzymes NADPH oxidases (NOXs) and downregulates nuclear factor E2-related factor-2 (Nrf-2) which is associated with multiple drug resistance in cancer. Further, we found that PMsCur inhibits Epithelial to Mesenchymal transition and attenuates invasive

capabilities of MCF-7/R cells. Interestingly, PMsCur was nontoxic to normal cells. Taken together, this study suggests that PMsCur drastically sensitize drug resistant MCF-7 cells and enhance therapeutic potential of curcumin with a safety profile, which may offer a new stratagem for improving drug delivery in cancer therapy.

P337/B344

The Antimalarial Drug Pyronaridine Interacts with DNA, Inhibits Topoisomerase II, and Is a Good Candidate for a Repurposed Anticancer Drug.

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Based on our previous work, the antimalarial drug pyronaridine (PND) was shown to exert anticancer activity. PND induces apoptosis by means of mitochondrial depolarization, alteration of cell cycle progression, and DNA intercalation. We have further investigated the mode of action of PND and have determined that it acts as a topoisomerase II inhibitor. In addition, preliminary *in-vivo* studies suggest that PND hinders tumor progression. In vitro combination studies of PND with known anticancer drugs such as Cisplatin and Gemcitabine, show that the combined effect of these drugs with PND have greater cytotoxic activity against cancer cells than individual administration. This results lead to the possibility of future *in- vivo* combination experiments. The findings presented in this study reconfirm and give a positive outlook to PND's potential as an anticancer drug.

P338/B345

Application of Anticancer Drug Screening System Using Silica Fiber-based Three-dimensional Culture Scaffold.

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[Purpose] Intrinsic or acquired multidrug resistance (MDR) in cancer cells is one of the major obstacles in the chemotherapeutic treatment of solid tumors. It is important to develop a cell-based in *vitro* system for testing anti-cancer drugs that can reflect the in *vivo* MDR characteristics of cancer cells. In this study, we examined the phenomenon of doxorubicin (DOX) resistance in human hepatoma (HepG2) cells by using the silica fiber-based three-dimensional (3D) culture scaffold (Cellbed) and compared the half maximal inhibitory concentration (IC_{50}) of DOX in 3D Cellbed culture with that of a monolayer culture. Furthermore, we examined DOX efflux in the 3D Cellbed culture. [Methods] After seven days of cultivation in the monolayer and 3D Cellbed cultures, the cells were exposed to DOX for two days. IC_{50} was measured by WST-8 assay with or without cyclosporin a (CsA), which is used to overcome drug resistance. Then we examined DOX efflux in the 3D Cellbed culture. After pre-incubation of the cells with 50 µM DOX at 37°C for 1 h, efflux was measured over time in the presence or absence of CsA. [Results and Discussion] the IC_{50} of DOX without CsA was significantly higher in the 3D Cellbed culture compared with that in the monolayer culture of HepG2 cells. However, the IC_{50} of DOX with CsA decreased to almost the same value in both the 3D Cellbed and monolayer cultures. These results suggest that the drug resistance phenomena of human hepatoma cells were expressed using the 3D Cellbed culture in *vitro*. We next examined the effects of CsA on DOX efflux from HepG2 cells in the 3D Cellbed culture. After 60 min, DOX efflux was markedly inhibited by CsA. This result suggests that reduction of the DOX IC_{50} in Cellbed culture by CsA reflects CsA-mediated inhibition of multidrug

resistance protein 1 (MDR1) activity. [Conclusion] the 3D Cellbed culture seems to provide a promising cell-based in *vitro* assay system for screening agents that may overcome the drug resistance of hepatic cancer cells.

P339/B346

Navitoclax Dramatically Enhances Death of Cells Arrested in Mitosis by Various Microtubule Inhibitors.

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Mitotic arrest induced by different microtubule inhibitors often results in the mitotic slippage with cell survival. This is the major limitation for the anti-cancer drugs based on the mitotic arrest. We analyzed the dose-dependent effect of microtubule inhibitors by time-lapse microscopy using two normal and six human cancer cell lines. Duration of mitotic arrest is almost independent from the drug concentration above the certain threshold, i.e. when no cell is able to undergo bi-polar or multi-polar division. On average it is in the range of 5-15 hours depending on the drug and cell type, and cells frequently undergo mitotic slippage. Slippage frequency remains relatively large even for high (μM) concentrations of Nocodazole, Vinorelbine and Paclitaxel. For each cell line examined the duration of mitotic arrest was the same for cells either dying in mitotic stage or undergoing mitotic slippage. Death of cells during mitotic arrest occurred through standard apoptotic pathway and activation of executive caspases 3/7 was rapid similarly to the Staurosporine-induced apoptosis. Cells had survived after mitotic slippage for more than 40 hours had long life expectancy. In attempt to minimize survival of cells arrested in mitosis we used Navitoclax (inhibitor of Bcl-2 proteins) together with microtubule inhibitors. Navitoclax at the concentrations of 0.3-1 μM was not toxic when applied alone, however induced apoptosis of cells after treatment with microtubule inhibitors. Combined treatment with microtubule inhibitors at concentrations inducing prolonged mitotic arrest and Navitoclax (300 nM) results in death of all cells had entered mitosis whenever they were unable to enter anaphase within 8-10 hours. Whereas, when cells undergo anaphase (though often abnormal one) in shorter time (4±2 hours), effect of Navitoclax diminishes. Flow cytometry analysis confirmed that accumulation of the G2/M population that happens after treatment with microtubule inhibitors in the concentration inducing prolonged mitotic blockade was diminished by addition of Navitoclax simultaneously with the increase of percentage of apoptotic cells. We suggest that inhibition of bcl-2 proteins makes mitotic cells more susceptible for apoptosis when cells are unable to switch from mitotic stage into interphase for a prolonged period of time. We conclude that Navitoclax is a potent inhibitor of mitotic slippage. This work was supported by Grant # AP05134232 from MES of the Republic of Kazakhstan.

P340/B347

MicroRNA-18a Targets MMP-3 in Platinum-resistant Ovarian Cancer.

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Ovarian cancer is the deadliest gynecological malignancy in the United States accounting for around 14 thousand deaths annually. Currently, platinum-based therapies remain as the standard treatment for ovarian cancer patients. However, a significant number of women become resistant to cisplatin treatment. Although several mechanisms of cisplatin resistance have been confirmed, therapies targeting these pathways have not been clinically effective. MicroRNAs (miRNAs) are key regulators of gene expression at the post-transcriptional and play a key role in ovarian cancer progression and drug resistance. To identify novel target miRNAs that may be involved in platinum resistance we performed a miRNA expression array between cisplatin-resistant and cisplatin-sensitive ovarian cancer cell lines. In this preliminary study, we found that miR-18a-5p (miR-18a) was downregulated in cisplatin-resistant ovarian cancer cells compared to the sensitive parental cells. Furthermore, the use of miR-18a oligonucleotide mimics (miR-18a-OMM) showed therapeutic potential in cisplatin-resistant ovarian cancer cells*. To* further evaluate the role of mir-18a in the cisplatin resistance of ovarian cancer we sought to identify the miR-18a potential target genes. We performed a bioinformatic analysis using miRNA target prediction programs and expression profiles of ovarian cancer cells. The experimental validation was conducted by qPCR, Western Blot and luciferase reporter assays. By qPCR we observed downregulation of seven potential target mRNAs upon miR-18a-OMM transfection compared to control-OMM transfected cells. However, at the protein level, we only observed downregulation in MMP-3 (Stromelysin-1). In addition, we validated the direct interaction of miR-18a with the 3'UTR of MMP-3 by luciferase reporter assay. We also observed that MMP-3 was significantly increased in cisplatin-resistant ovarian cancer cells compared to their sensitive counterparts, the opposite expression trend observed for miR-18a. These results demonstrate that MMP-3 is a direct target of miR-18a in cisplatin-resistant ovarian cancer.

P341/B348

Potential Cancer Therapy by the Development of a Protein-based Drug Delivery System Nanoparticles Using Plant-derived Triterpenoids as Drugs.

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Nano-formulations for cancer therapy have become a strategy to minimize the lack of drug inactivation after administration into patients. An alternative to increasing the effectiveness of drugs is by the development of nanoparticles as drug delivery systems. Consequently, protein-based nanoparticles have been studied due to their potential to simultaneously increase the drug solubility and biological targetability for cancer therapy. Within the compound with solubility difficulty are the triterpenoids, which have shown anticancer activity against cancer. Herein, we developed spherical protein-based nanoparticles (diameter size 160-250 nm and polydispersity index value from 0.13) by use of a micro-emulsion method. Our nanoparticles sizes are compatible with tumor accumulation through the irregular vasculature observed in cancer tissues. Our system demonstrated toxicity in a cancer cell line, after incubation for 24h.

P342/B349

Down Regulation of Abc Transporters by the Erythropoietin Treatment Accelerates the Affect of Anti-cancer Reagents.

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Erythropoietin (EPO) is a glycoprotein cytokine which stimulates red blood cell production (erythropoiesis) in the bone marrow. The receptor of it (EPOR) was reported to exist on the cancer cellular membrane: more than 85% cancer cell expresses EPOR. Several reports suggested to play an important role for cancer cellular growth, however, the role of EPOR in cancer cells is not confirmed. The authors have studied the cancer cellular porphyrin accumulation, metabolism and excretion to clear the pathogenetic mechanism which involved the high concentration of porphyrins. The authors found that reactive oxygen species (ROS) accelerate both the cancer cellular porphyrin accumulation and the effect of photodynamic therapy. However, we cannot confirm which substrate is a key of signal transduction. EPO has been known used to be up-regulated at the metabolic acidosis via the stabilization of HIF-1α, a nuclear factor. Since HIF-1α can be stabilized by not only acidosis but also the high concentration of nitric oxide (NO), NO rich cancer cells may involve EPOR. Moreover, the purpose of EPO-EPOR system is likely to survive the hypoxic condition, it may keep intracellular heme/ porphyrins. The authors' hypothesis is EPO-EPOR system in cancer cells may down-regulate ABCG2 which excretes heme/ porphyrins, and several anti-cancer drugs. Moreover, if so, the excretion of several anti-cancer chemo reagents should be inhibited to involve more effective chemotherapy. Methods: to prove these possibilities, the authors carry out following experiments with gastric and colorectal cancer cells. The existence of EPOR, the amount of ABC transporters (B1, C5, G2) after the recombinant EPO (rEPO) exposure (dose dependency and time dependency) were analyzed by western blotting. Cytotoxicity of anti-cancer drugs with or without rEPO was measured with the Cell Counting Kit-8. Result: (1) EPOR existed in each cell line used in this study. (2) rEPO treatment involved down regulation of ABC transporters (B1, C5, G2) in dose dependently. (3) ABCG2 expression decreased until 24 hours after rEPO treatment. However, its expression re-increased 48 hours after treatment. (4) the 24 hours pretreatment of EPO significantly increased the effect of doxorubicin, paclitaxel, irrinotecan, cisplatin, 5-FU and oxaliplatin even in chemo-resistant cells. Conclusions: rEPO accelerates the effect of anti-cancer drugs via the down regulation of ABC transporters.

P343/B350

Lopinavir and Curcumin Directly Alters Bax/bcl2 and Vegf165b Mrna Levels to Suppress Human Squamous Cervical Carcinoma Cell Growth.

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Following the success of the highly active antiretroviral therapy, the potential of a multidrug combination regimen for the management of cancer is intensely researched. The anticancer effects of curcumin on some human cell lines have been documented. Lopinavir is an FDA approved protease inhibitor with known apoptotic activities. Dysregulated apoptosis is important for the initiation of cancer while angiogenesis is required for cancer growth and development, this study, therefore, investigated the effects of the combination of lopinavir and curcumin on cell viability, apoptosis and the mRNA expression levels of key apoptotic and angiogenic genes; BAX,BCL2 and VEGF165b in two human cervical

cell lines; human squamous cell carcinoma cells - uterine cervix (HCS-2) and transformed normal human cervical cells (NCE16IIA). The two human cervical cell lines were treated with physiologically relevant concentrations ofthe agents for 120 h following which BAX, BCL2, and VEGF165b mRNA expression were determined by Real-Time qPCR. The Acridine Orange staining for the morphological evaluation of apoptotic cells was also performed. The combination of lopinavir andcurcumin up-regulated pro-apoptotic BAX and antiangiogenic VEGF165b but down-regulated the mRNA levels of anti-apoptotic BCL2mRNA in the human squamous cell carcinoma (HCS-2) cells only. The fold changes were statistically significant. Micrographs from Acridine Orange staining showed characteristic evidence of apoptosis in the human squamous cell carcinoma (HCS-2) cells only. The findings reported here suggest that the combination of curcumin and the FDA approved drug-lopinavir modulate the apoptotic and angiogenic pathway towards the inhibition of cervical cancer.

P344/B351

The Anti-proliferative Effect of the Newly Hybrid Compound against Human Cervical Cancer Involves Apoptosis Mediated by Stat3 Inactivation.

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We previously reported the potential anti-proliferative activity of 3-(5,6,7-trimethoxy-4-oxo-4H-chromen-2-yl)-N-(3,4,5-trimethoxyphenyl)benzamide (TMS-TMF-4f) against human cancer cells; however, the underlying molecular mechanisms have not been investigated. In the present study, TMS-TMF-4f showed the highest cytotoxicity in human cervical cancer cells (HeLa and CaSki) and low cytotoxicity in normal ovarian epithelial cells. An nexin V-FITC and propidium iodide (PI) double staining revealed that TMS-TMF-4f-induced cytotoxicity was caused by the induction of apoptosis in both HeLa and CaSki cervical cancer cells. The compound TMS-TMF-4f enhanced the activation of caspase-3, caspase-8, and caspase-9 and regulated Bcl-2 family proteins, which led to mitochondrial membrane potential (MMP) loss and resulted in the release of cytochrome *c* and Smac/DIABLO into the cytosol. Also, TMS-TMF-4f suppressed both constitutive and IL-6-inducible levels of phosphorylated STAT3 (p-STAT3) and associated proteins such as Mcl-1, cyclin D1, survivin, and c-Myc in both cervical cancer cells. STAT-3 overexpression completely ameliorated TMS-TMF-4f-induced apoptotic cell death and PARP cleavage. Docking analysis revealed that TMS-TMF-4f could bind to unphosphorylated STAT3 and inhibit its interconversion to the activated form. Notably, intraperitoneal administration of TMS-TMF-4f (5, 10, or 20 mg/kg) decreased tumor growth in a xenograft cervical cancer mouse model demonstrated by the increase in TUNEL staining and PARP cleavage and the reduction in p-STAT3, Mcl-1, cyclin D1, survivin, and c-Myc expression levels in tumor tissues. Taken together, our results suggest that TMS-TMF-4f may potentially inhibit human cervical tumor growth through the induction of apoptosis via STAT3 suppression. This research was supposed by Basic Science Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1D1A1B04030534).

P345/B352

Investigation of New Podophyllotoxin Derivatives as Anti-cancer Drug.

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We previously reported that podophyllotoxin acetate (PA) inhibits the growth and proliferation of non-small cell lung cancer (NSCLC) cells and also makes them more sensitive to radiation and chemotherapeutic agents. In an attempt to enhance PA activity, we synthesized 34 derivatives based on podophyllotoxin (PPT). Screening of the derivative compounds for anti-cancer activity against NSCLC led to the identification of β-apopicropodophyllin (APP) as a strong anti-cancer agent. In addition to its role as an immunosuppressive regulator of the T-cell mediated immune response, the compound additionally showed anti-cancer activity against A549, NCI-H1299 and NCI-460 cell lines with IC50 values of 16.9, 13.1 and 17.1 nM, respectively. The intracellular mechanisms underlying the effects of APP were additionally examined. APP treatment caused disruption of microtubule polymerization and DNA damage, which led to cell cycle arrest, as evident from accumulation of phospho-CHK2, p21, and phospho-Cdc2. Moreover, APP stimulated the pro-apoptotic ER stress signaling pathway, indicated by elevated levels of BiP, phospho-PERK, phospho-eIF2α, CHOP and ATF4. We further observed activation of caspase-3, -8 and -9, providing evidence that both intrinsic and extrinsic apoptotic pathways were triggered. In vivo, APP inhibited tumor growth of NSCLC xenografts in nude mice by promoting apoptosis. Our results collectively support a novel role of APP as an anticancer agent that evokes apoptosis by inducing microtubule disruption, DNA damage, cell cycle arrest and ER stress.

Gene Structure and Transcription

P346/B354

Translational Profiling of UBQLN2 Mouse Models of ALS/FTD Reveals Novel Insight into Disease Progression.

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Amyotrophic lateral sclerosis (ALS) is a fatal, neurological disease characterized by the progressive degeneration and subsequent death of both upper and lower motor neurons. Neuronal-specific expression of ALS/FTD-linked P497S Ubiquilin 2 (UBQLN2) mutation generates disease pathology in mice, which mimics the familial human disease. Traditionally, glial cells - including astrocytes - are known static supporters of neurons in the CNS. More recently, astrocytes have been implicated as integral components of nervous system networks and have been shown to proliferate disease progression. Here, we used translating ribosomal affinity purification (TRAP) to isolate motor neuron and astrocyte cell-type-specific mRNA from brain and spinal cord of 14 and 21 week old mice. Through translational profiling and high-throughput mRNA sequencing, age-related transcription level changes in astrocytes and motor neurons have been identified. Despite neuron-specific expression of the mutant P497S UBQLN2 protein, initial observations using Ingenuity Pathway an alysis (IPA) indicate significant changes in astrocytes - most notably the inhibition of oxidative phosphorylation. Thus, neuron-specific P497S UBQLN2 mutation initiates significant disruption in astrocytes, indicating that they may play significant role in pathogenesis. Comparison analyses between translatome and proteome data has provided novel insight that should enable us to discover drivers of disease progression.

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A Role of Physical Properties of DNA in the Transcription of Non-coding RNA.

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We previously reported that a highly distinctive mechanical property resides in the majority of human pol II promoters and it plays a central role in the promoter function (Fukue *et al*.,*Nucl. Acids Res*., 2004, 2005). In the last decade, promoter databases were remarkably upgraded. Thus, we recently performed a follow-up study using both coding and non-coding RNA promoters. First, we classified the target regions (-200 to +200 relative to the TSS) into two groups: those carrying a core promoter element such as TATA-box, Inr, DPE, etc. An d those without any such element ("core-less promoters"). The former group was further divided into subgroups according to the element species. Second, the average flexibility profile was calculated for each group. Regarding the pol. II promoters, our original finding was confirmed. However, for the core-less non-coding RNA promoters, their average flexibility profile was somewhat different from that of the corresponding pol. II promoters: although the profiles around the TSS were similar with each other, ~+30 region also showed a distinctive flexibility profile in the case of core-less non-coding RNA promoters.To scrutinize the data, we further performed clustering with unsupervised machine learning. As the result, the core-less group was found divided into four clusters each with their own flexibility profile. Interestingly, it was also clarified that the average promoter strengths are considerably different among the four. Furthermore, the properties such as duplex disruption energy and protein-induced deformability were also found different among them. These results suggest that physical properties of DNA also play a key role in the function of the core-less non-coding RNA promoters and determine their relative strengths.

P348/B356

Colocalization of Cruciform Motifs and the Mammalian Poly(A) Signals.

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Genomic DNAs have a large number of inverted repeat (IR) sequences. However, their function, if any, still remains elusive. Recently, we reported that IRs with cruciform-forming potential (referred to as 'CFIRs') are actively placed in the termination region of transcription in the *Escherichia coli* and *Saccharomyces cerevisiae* genomes (Miura *et al.*, *Curr. Genet.*, 2018, 2019). Furthermore, we also reported that there is a strong structural correlation between CFIRs and poly(A) signals of *S. cerevisiae* and the CFIRs generally exclude nucleosomes from the regions (Miura *et al.*, *Curr. Genet.*, 2019). In the current study, using the genomes of mouse and humans, we performed genome-wide analyses for the distribution, occurrence frequency and sequence characteristics for CFIRs. We found that A-tract- or T-tract-rich CFIRs were highly enriched on and around the poly(A) sites in both cases. Interestingly, the distribution profiles of CFIRs were somewhat similar among *E. coli*, *S. cerevisiae*, mouse and humans, suggesting their common role in the transcription termination. Furthermore, a strong structural correlation was found between the CFIRs and the poly(A) signals of mouse and humans. We also examined the relationship between CFIRs and chromatin structure in mouse. It suggested that the majority of the CFIRs occurring on and around the poly(A) sites is incorporated into nucleosomes and determines rotational setting of DNA, which is different from the case of the yeast. In this meeting, we will discuss what the colocalization of the cruciform motif and the poly(A) signal suggests.

P349/B357

Role of Curi Complex in Regulation of Mtorc1 Target Genes under Stress and Starvation.

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Ribosome biogenesis is an expensive process involving expression of ribosomal protein genes and ribosomal RNA, consuming up to 90% of a cell's energy. Thus, this serves as a premier biological process to be targeted in emergencies such as stress and starvation to save energy. In yeast, Hmo1p (a high mobility group protein) along with other proteins binds Ribosomal Biogenesis (RiBi) genes and RNA Pol I transcribed genes. Besides RiBi-genes, Hmo1p also binds its own promoter. Interestingly, expression of *HMO1* is suppressed in response to mTORC1 (target of rapamycin complex) inhibition by rapamycin. Inhibiting mTORC1 causes the transcription factor Ifhl1p to leave the *HMO1* promoter. Ifhl1p moves to the nucleolus and forms another complex with Utp22p, CK2p and Rrp7p, a complex named as CURI. This complex formation seems to have a synergistic function in decreasing Ribosomal Biogenesis because Utp22p and Rrp7p are also involved in pre-ribosomal RNA processing when Ifhl1 is absent from the nucleolus. We report here that rapamycin treatment also decreases expression of *UTP22 and RRP7* up to five-fold. Since Ifhl1p gets replaced by the repressor Crf1p at the *HMO1* promoter during nutritional stress, we conducted a ChIP-assay at the *UTP22* and *RRP7* genes. Preliminary data from ChIP-assay suggests inactivation of *UTP22 and RRP7* gene expression by a mechanism similar to *HMO1* suppression; we detected replacement of Ifhl1p by Crf1p at the *UTP22* and *RRP7* promoters after 15 minutes. While reduced *HMO1* expression in response to rapamycin requires Tor1p, deletion of *TOR1* did not markedly affect expression of *UTP22* or *RRP7* on addition of rapamycin. We therefore propose that Tor2p can substitute for Tor1p in mTORC1 for regulation of *UTP22 and RRP7*, but not for regulation of *HMO1*.

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Alternate Approaches to Understand Regulatory Mechanisms behind the Expression of a Sodium Calcium Potassium Exchanger in *Caenorhabditis Elegans*.

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Neurons utilize Na^+ and Ca^{2+} ions to regulate excitability. Defects in exchange can lead to a variety of health problems, such as epilepsy, which is characterized by an onset of spontaneous, recurrent seizures, arising from a loss in the balance of excitatory and inhibitory nerve impulses. One of the primary mechanisms by which cells maintain balanced levels of these $Na^{+\text{ and }}Ca^{2+}$ ions is by utilizing Na^+/Ca^{2+} Exchangers (NCX). Very little is known about the regulation of *ncx* genes, of which the *nckx* sub-family remains particularly under-studied. To learn more about the transcriptional regulation of this gene family, I study an *nckx* expressed in *C. elegans* named NCX-5. *ncx-5* expression is limited to a specific circuit of six sensory neurons. All six neurons are gas sensing the goal of my research is to understand why *ncx-5* expression is limited to a specific sensory circuit. I performed a forward genetic screen to uncover determinants of *ncx-5* transcriptional regulation. The screen yielded a mutant containing very faint expression. We are currently attempting to identify the underlying lesion within this mutant. In order to study cis-regulation of *ncx-5*, I dissected the *ncx-5* promoter sequence into two 700bp portions. I fused each portion to the coding sequence for Green Fluorescent Protein (GFP) and generated transgenic animals expressing these truncated promoter::GFP fusions. One of the constructs yielded a transgenic animal with GFP expression in all six sensory neurons, suggesting that this region of the promoter contains the minimal regulatory motif required to activate the wildtype *ncx-5* expression.

In order to find this motif I further dissected this 700bp region and have now identified a 78bp region of the promoter, which is sufficient to activate *ncx-5* expression only in the six sensory neurons. I conducted bioinformatics analysis using JASPAR and identified five transcription factors that potentially bind to this region. The next step is to obtain mutants for these proteins and cross them into a transgenic strain expressing ^{prom}*ncx-5*::GFP. Phenotype of the second generation progeny will be observed under fluorescence to detect any variation. These animals will also be genotyped to verify inheritance of mutation. In addition, I am currently generating mutants of *ncx-5* utilizing the CRISPR-Cas9 system. The mutation is designed to knock out expression of the gene, allowing us to test any resulting effects on behaviour, such as gas sensation and feeding abilities. A combination of the promoter dissection protocol and the forward genetic screen will allow us to develop a better understanding of how this family of proteins are regulated. Obtaining a null mutant for *ncx-5* via CRISPR will help us understand more about the function of *ncx-5 in* the gas sensing circuit it is expressed in.

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NF90 and NF45 Are Chromatin Regulators of Inducible Gene Expression.

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NF90 and its heterodimeric partner protein NF45 are nucleic acid-binding proteins that have been established to regulate RNA splicing, stabilization and export. The roles of NF90 and NF45 in chromatin regulation of gene transcription has not been comprehensively characterized. We used chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) identified 9,081 genomic sites specifically occupied by NF90 in K562 cells. One third of NF90 peaks occurred at promoters of annotated genes. NF90 occupancy colocalized with chromatin marks associated with active promoters (H3K9ac) and strong enhancers (H3K27ac). Comparison with 150 ENCODE ChIP-seq experiments revealed that NF90 clustered with transcription factors exhibiting preference for promoters over enhancers (*POLR2A*, *MYC*, *YY1*). Integrating NF90 ChIP-seq data with differential gene expression analysis following shRNA knockdown of NF90 in K562 cells revealed that NF90 regulated expression of immediate early genes *EGR1*, *FOS*, and *JUN*. In HEK293 cells, serum starvation followed by stimulation with Phorbol 12-myristate 13-acetate (PMA) induces dynamic and reciprocal binding of NF90 and NF45 at promoters of these immediate early transcription factors. In cells stably transfected with doxycycline-inducible shRNA vectors targeting NF90/ILF3 or NF45/ILF2, doxycycline-mediated knockdown of NF90 or NF45 attenuated the inducible expression of *EGR1*, *FOS*, and *JUN* at the levels of transcription, RNA and protein. Taken together, we present evidence that NF90 and NF45 are constitutively expressed transcription factors pre-existing at the promoters of immediate early genes. Dynamic and reciprocal chromatin binding of NF90 and NF45 at immediate early promoters are observed upon stimulation, and NF90 and NF45 contribute to inducible expression of immediate early genes. NF90 and NF45 may thus serve as chromatin regulators of the immediate early response.

P352/B360

New Emergent Properties of Transcription Factor Dynamics and Their Interactions with Chromatin.

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Single-molecule tracking allows the study of transcription factor dynamics in the nucleus, giving important information regarding the search and binding behavior of these proteins with chromatin in *vivo*. However, these experiments suffer from limitations due to photobleaching of the traced protein and pre-assumptions on exponential behavior required for data interpretation, potentially leading to serious artifacts. Here, we developed an improved method to account for photobleaching effects, theory-based models to accurately describe transcription factor dynamics, and an unbiased model selection approach to determine the best predicting model. A new biological interpretation of transcriptional regulation emerges from the proposed models wherein transcription factor searching and binding on the DNA and nuclear microenvironment heterogeneity result in a broad distribution of binding affinities and accounts for the power-law behavior of transcription factor residence times. Moreover, two types of confinement are discovered; one related with specific DNA interaction and a second one related to liquid-liquid phase separation. The latter shows the importance of phase separation in transcriptional regulation and gene expression.

P353/B361

The Quaternary Structure of the Mineralocorticoid Receptor Depends on Ligand and Dna Binding.

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The mineralocorticoid and glucocorticoid receptors (MR and GR) are evolutionarily related nuclear receptors with high sequence conservation and a shared hormone response element (HRE). Both receptors are activated by glucocorticoids, but MR can be selectively activated by aldosterone. It has recently been proposed that liganded GR dimers form tetramers upon DNA binding in live cells. We show that liganded MR also adopts higher quaternary arrangements in live cells, using the imaging technique Number & Brightness (N&B). MR adopts a tetrameric organization in the nucleoplasm and forms complexes with an average of 7 receptor units upon binding an HRE. Interestingly, MR antagonists eplerenone and spironolactone induced intermediate oligomerization arrangements, strongly suggesting that higher order oligomerization is essential for receptor activity. With corticosterone, GR can incorporate into MR complexes partially displacing MR monomers. Genome-wide gene expression and receptor binding studies suggest corticosterone liganded MR and GR in the same cells have potentially complex functional interactions, contributing to receptor-specific signaling. Certain genes respond to only one receptor while others respond to both receptors. The interaction of these two closely-related receptors has important implications for the mechanisms for glucocorticoid signaling and transcription factors in general.

P354/B362

Allelic Exclusion of Telomeric Variant Surface Glycoprotein Genes in Trypanosomes Regulated by Phosphoinositides.

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The mechanisms by which eukaryotes control allelic exclusion remain poorly understood. The single-celled protozoan pathogen *Trypanosoma brucei* exclusively expresses one variant surface glycoprotein (VSG) gene at a time out of ~ 2,500 VSG genes, and VSG transcription occurs at one of the 20 telomeric expression sites (ESs). *T. brucei* periodically changes VSG expression by transcriptional switch between ESs or VSG gene recombination, and this process is essential for *T. brucei* evasion of the host antibody response by antigenic variation. We identified a telomeric ES multiprotein complex that controls allelic exclusion of VSG genes. Its activity is regulated by a phosphoinositide system which involves a phosphatidylinositol phosphate 5-phosphatase (PIP5Pase) enzyme that binds repressor activator protein 1 (RAP1). Knockdown of PIP5Pase results in simultaneous transcription of all telomeric and subtelomeric VSG genes. PIP5Pase dephosphorylates PI(3,4,5)P3 which prevents this metabolite interaction with RAP1, and thus preserves RAP1 association to ES chromatin and results in transcriptional repression of VSG genes. Catalytic mutations of PIP5Pase that inhibit PI(3,4,5)P3 dephosphorylation affect RAP1 association with ES chromatin and result in VSG transcription, i.e., loss of allelic exclusion. This mechanism, which is also essential for silencing VSG gene transcription during parasite development from mammalian infectious to non-infectious stages, might be conserved in other eukaryotes.

P355/B363

Gamma-tubulin Gene Intron Length Polymorphism of Different *Camelina* Species.

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Different species from the genus *Camelina* could be considered as promising sources for oil production. One of them, cultivated species of false flax (*C. sativa* L. Crantz.) have been the focus of many investigations to date, but other representatives of this genus are underused despite their potential utility as a germplasm donors for *C. sativa* genetic improvement. In addition, a limitation on broader use of *C. sativa* as a genetic model and for gene editing is its hexaploid nature. In this study, DNA profiling of six *Camelina* species (*C. microcarpa*, *C. rumelica*, *C. hispida* var. *grandiflora*, *C. Alyssum*, *C. laxa and C. sativa*) was conducted basing on assessment of γ-tubulin intron length polymorphism. Because only two copies of γ-tubulin gene are represented in a diploid genome, ploidy can also be evaluated with this method. PCR was performed using degenerate primers, and amplification products were analyzed electrophoretically in a non-denaturing acrylamide gel. Among seven studied *C. microcarpa* accessions obtained from USDA (http://www.ars-grin.gov/), two samples (PI650134, PI650135) possessed atypical γ-tubulin intron patterns. *C. microcarpa* had 4 amplicons of 507 bp, 528 bp, 557 bp, 620 bp. The PI650134 accession contained four amplicons, two being similar to *C. rumelica* - 510 bp and 578 bp, as well as 553 bp and 700 bp fragments. The PI650135 accession had two fragments only - 507 bp and 600 bp, which are unique compared to other studied *Camelina* species. Molecular genetic analysis using SSR markers confirmed differences among the six camelina species. Previously, Galasso et al. (2018) described accessions characterized by atypical genetic profiles based on β-tubulin intron length polymorphism. Brock et al. (2019) described the PI650135 accession as a separate species *C. neglecta*. γ-Tubulin intron profiles of *C. Alyssum and C. sativa* were very similar to *C. microcarpa*, which could be

explained by their common origin. In *C. rumelica,* two fragments of 510 bp and 578 bp were found, while *C. hispida* possessed high number of fragments (about 10) of 498-956 bp length. Together, these results provide evidence of high polymorphism of this species. Based on our findings, we can conclude that *C. neglecta and C. rumelica* are diploid species and, as such, their use could simplify transformation or genome editing of the hexaploid *C. sativa*.

P356/B364

A Functional Intronic Minisatellite VNTR 2-1 in Human Telomerase Gene Regulation.

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Abstract Telomerase is critical for telomere maintenance in human stem cells and cancer cells but silenced in most somatic cells. Intronic regions of the human telomerase gene (hTERT), but not those of the mTert gene, play an important role in hTERT expression. Here we demonstrated that, VNTR 2-1, a 42bp/unit minisatellite located in the hTERT intron2 acts as an enhancer element that regulates hTERT promoter activity. Our data showed that 2-32 VNTR 2-1 repeats were able to induce the luciferase activity of pGL3 reporter vectors; and histone mark H3K4me2 was detected on its endogenous locus in K562 cells indicating the potential location of enhancers. By searching from public ChIP-seq database, several bHLH family transcription factors were found bind to the genomic region of hTERT VNTR 2-1 that possibly through interaction with the Ebox motifs. Consequently, we hypothesized that VNTR 2-1 contains functional *cis*-regulatory elements that recruit transcription factors (TFs) to facilitate human-specific regulation of the telomerase gene. To test this hypothesis, we first verified the functional role of Ebox clusters presented in VNTR 2-1 repeats using reporter assay. The Ebox mutation constructs lost over 90% of luciferase activity compare to the WT VNTR 2-1 constructs. Because there were numerous publications verified the interaction of bHLH family proteins with Ebox motifs, two bHLH TFs, USF1 and MNT, were selected for further study. As shown by the qRT-PCR and hTERT BAC reporter, USF1, dimerized with USF2 can upregulate hTERT expression whereas MNT repressed this activity. This is consistent with their general role in gene transcription. Future experiments will focus on identifying new transcription factors and validating their functions toward hTERT expression. This research will gain the understanding of genomic repetitive sequence as well as the molecular mechanisms of telomerase regulation. Key words: VNTR, hTERT regulation, intronic enhancer, transcription factors

P357/B365

Deletion of the CDK Mediator Subunit Cdk8 Exhibits a Memory Effect on *HAP4* Expression.

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The transcription factor Hap2/3/4/5 is a master regulator of respiratory gene expression and mitochondrial biogenesis in *Saccharomyces cerevisiae*. The activity of this complex is largely dependent on the protein level of Hap4, which is regulated in part by the Mediator complex, a highly conserved transcriptional coactivator that interacts with RNA polymerase II. In yeast, the 25 subunits of Mediator are divided into four modules that play differential roles on gene expression. While the Head, Middle, and Tail modules have been shown to positively regulate transcription, evidence suggests that the CDK module is a negative regulator of gene expression. Mutants of the CDK module subunits Med12, Med13, Cdk8, and CycC have induced expression of *HAP4*. Deletion of Cdk8 exhibits a "memory" effect on *HAP4* expression. When a *HAP4-lacZ* reporter gene is introduced to *cdk8Δ* mutants, there is a low induction (LI) of *HAP4* expression compared to wild type. However, when the *cdk8Δ* gene deletion is introduced

into wild-type cells already carrying the *HAP4-lacZ* reporter gene, there is a high induction (HI) of *HAP4* expression. The "memory" effect of *HAP4* expression in *cdk8Δ* is also found in *med12Δ and med13Δ,* but not in CycC mutant cells. *HAP4* expression in the *cdk8Δ* LI and HI strains remains stable after many generations, with a very low frequency of conversion from the high to low induction phenotype, but not the reverse. The "memory" effect is also maintained in the diploid strains when crossed with either wild-type or *cdk8Δ* deletion strain. Similar results were found for *med12Δ and med13Δ* mutants after crossings*. To* address the mechanism responsible for maintaining "memory", we introduced gene mutations involved in histone modification and chromatin remodeling, namely, *htz1Δ, gcn5Δ, and ldb7Δ*, *in to cdk8Δ* LI and HI strains. *gcn5Δ and ldb7Δ* lead to reduced *HAP4-lacZ* expression in HI and LI *cdk8Δ* mutants to a similar extent. Deletion of the histone H2A variant *HTZ1 in* HI *cdk8Δ* strain led to the low induction phenotype. In LI *cdk8Δ* strain, *htz1Δ* did not change the low induction phenotype of *HAP4* expression. Interestingly, *htz1Δ in* wild-type strain resulted in a low induction of *HAP4-lacZ* expression. Our results suggest that this novel "memory" phenotype of altered *HAP4* expression due to *cdk8Δ* is epigenetic and the result of changes in chromatin structure.

P358/B366

Nuclear Actin in Transcription by RNA Polymerase II.

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Actin is a protein that exists in cells both as monomers and as filaments. Although widely known as a major component of the cytoskeleton, actin also localizes to the nucleus, where it has important roles especially related to gene expression. Actin is a part of several chromatin remodeling complexes and takes part in pre-mRNA processing. Intriguingly, actin has also been linked to transcription in various ways and nuclear actin levels appear to be actively regulated to sustain maximal transcription. Actin co-purifies with all three RNA polymerases and has been suggested to interact with positive transcription elongation factor b (P-TEFb) essential in pause-release of Pol II. However, the biochemical details of these interactions are not known and this work focuses on characterizing them using a variety of tools. Our ChIP-seq results show a co-occupancy of actin and Pol II on transcription start sites of most transcribed genes as well as on the gene bodies of highly expressed genes and current experiments are focusing on the biochemical details of the possible interaction between actin and whole Pol II. Interestingly, our mass spectrometry screen for nuclear actin binding partners contained P-TEFb and a set of general transcription factors. Further characterization of mass spec hits showed that Cdk9, the kinase subunit of P-TEFb, binds actin monomers directly in biochemical experiments and inhibits actin polymerization and this applies to the whole P-TEFb complex as well. Actin and Cdk9 also interact in co-immunoprecipitation experiments. As most of P-TEFb is in cells bound to an inhibitory complex 7SK snRNP, ongoing experiments are now assessing the possibility that actin could help releasing P-TEFb from the inhibition, thus allowing it to act in the pause-release. In conclusion, these results support the positive role of nuclear actin in transcription.

P359/B367

Investigating the Coordination of Global Transcriptional Scaling with Cell Size and Growth.

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Cell size varies widely amongst cell types or environmental conditions, and during the life cycle of a proliferating cell its size will increase, on average, twofold. However, how the basic biosynthetic process of the cell, such as transcription, adapt to account for changes in volume has remained poorly understood. More specifically, it was first described in the 1970s that as cell increase in size the rate of RNA-synthesis per gene increases in proportion to cell size - this size-scaling of RNA-synthesis ensures RNA and protein concentrations are kept constant and is likely critical for maintaining biochemical reaction rates and setting the cellular growth rate. Despite the apparent importance of this phenomenon, the molecular basis of this size-scaling control has remained unexamined. Using a combination of ChIP-seq, chromatin-proteomics, and super-resolution (PALM) single-molecule imaging, we have determined that the RNA polymerases occupancy per gene increases across the genome as cell size increases. Furthermore, this increase is not correlated with any major change in histone occupancy or chromatin accessibility. Consistent with this, most histone modifications measured are unchanged with the exception of those downstream of the elongating polymerase. Nearly all transcription factors examined, including the major subunits of the RNAPII Pre-Initiation complex (PIC), increase their fractional occupancy on the genome in proportion to size, concomitant with the increased polymerase occupancy. Given that we have also observed that the amount of polymerase and transcription-factor molecules per cell is strongly correlated with cell size, we considered whether a critical subunit of PIC was limiting for transcription such that as the amount of this factor increases so would global transcription rates. To test this idea, we have measured polymerase occupancy after local perturbation of the nuclear concentrations to PIC components. Based on these data we propose a working model where global transcription rates are set via the combinatorial amount of multiple different PIC subunits - each of which is partially limiting.

P360/B368

Coordination of Protein Homeostasis with Cell-volume in Budding Yeast.

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In proliferating cells, protein homeostasis is tightly coordinated with cell-volume and most proteins are kept at a constant, cell-volume independent concentration. While well suited for most proteins, a constant concentration imposes a problem for DNA-binding proteins such as histones, which on the contrary are required at a constant amount. Indeed, using live-cell fluorescence microscopy, we find that in budding yeast the histone concentration decreases with increasing cell-volume. This raises the question of how cells achieve to produce histones in proportion to DNA content even though total protein production increases with cell-volume. To identify the cell-volume dependence of histone transcript concentrations, we controlled cell-volume through inducible expression of the G1/S inhibitor Whi5. Using RT-qPCR, we find that for control genes the mRNA concentration relative to total RNA stays constant with increasing cell-volume. In contrast, the relative concentration of histone mRNA decreases in inverse proportion to cell volume. Next, we asked whether histone promoters are sufficient to establish the cell-volume-dependence of histone homeostasis. Using RT-qPCR and flow cytometry, we find that histone promoters driving expression of a fluorescent reporter result in a decrease of reporter

mRNA concentration with increasing cell-volume, as opposed to the *ACT1* promoter, which leads to a constant, cell-volume independent, mRNA concentration. In conclusion, we find that histone transcription is limited by the DNA template, which is in contrast to the production of other proteins such as Act1, where transcription is limited by the transcriptional machinery. In addition, such a mechanism also explains the differential ploidy dependence of transcription driven by histone and control promoters. Thus, our work identifies a novel regulatory mechanism that enables cells to couple histone production to ploidy rather than cell size.

P361/B369

Function of the IR Cluster Located in the Upstream of the *Oct3/4 Ge*ne.

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Many studies have been carried out to understand the function of inverted repeat (IR) sequences that read the same from 5' to 3' in either strand. IRs are also referred to as palindromes. The function of IRs, however, is still elusive. The mouse *Oct3/4* gene, which is known as a key gene for pluripotency, has a cluster of six IRs in ~1.5 - ~2 kb the upstream. Each of them has a potential to transform into a cruciform. It is known that expression of the *Oct3/4* gene is regulated by its proximal promoter (PP), proximal enhancer (PE), and distal enhancer (DE). Considering that the DE harbors the IR cluster, this cluster may play some important role in the *Oct3/4* expression. In order to substantiate this hypothesis, applying the CRISPR/Cas9-based genome editing to the mouse genome in the ES cells, the IRs in the cluster were replaced with mirror repeat sequences that retain the sequences of the repeat unit in the respective IRs. In the resulting cells, these mutations slightly decreased the *Oct3/4* expression, while the transcription levels of the noncoding RNAs originated from the DE (i.e., DE-ncRNAs) were increased. In addition to this, expression of the *Sox2*, *Klf4*, *Nanog*, and *Esrrb* genes showed a similar change to that of the DE-ncRNAs expression. These results suggest that the IR cluster has a positive effect on the *Oct3/4* expression and a negative effect on the expression of the *Sox2*, *Klf4*, *Nanog*, and *Esrrb* genes through the expression of the DE-ncRNAs. In this meeting, we will discuss the putative function of the IR cluster.

P362/B370

Development of CRISPR Tool Cell Lines for Targeted Gene Expression Study.

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Developing technologies for targeted disruption of gene expression will provide powerful tools for studying gene function. To date, various methods for achieving loss-of-function (LOF) outcomes have been developed, including approaches employing RNA interference (RNAi) and CRISPR (clustered regularly interspaced short palindrome repeats)/Cas9 mediated gene perturbation (Nature Biotech (2016) 34(6): 631-634). In mammalian cells, RNAi is the predominant method for programmed knockdown of mRNAs, but its utility is limited by confounding off-target effects (Nat. Methods (2006) 3:777-779). The RNA-guided CRISPR-associated nuclease Cas9 provides an effective means of introducing targeted LOF mutations in the genome. Cas9 can be programmed to induce DNA double strand breaks (DSBs) at specific genomic loci through guide RNAs (gRNA), which when targeted to coding regions of genes, can create frame shift indel mutations resulting in a LOF allele (N Engl J Med (2019) 380(25): 2475-2477). In addition to the use of the nuclease activity of Cas9, the CRISPR-Cas9 technology can also be repurposed as a sequence-specific, non-mutagenic gene regulation tool. Coupling of the

engineered nuclease-deficient Cas9 (dCas9) to a transcriptional repressor domain can robustly silence expression of endogenous genes with high specificity, resulting in 'CRISPR interference' (CRISPRi) (Cell (2013) 152(5): 1173-1183). Here we report the creation of Cas9 expressing HEK293 and CRISPRi A549 cell lines, in which the Cas9 or KRAB-dCas9 expression cassette was integrated into AAVS1 safe harbor locus. The integration of knock-in allele was confirmed at genomic and translational levels in both cell lines. When gRNAs targeting p53 and RFP genes were delivered into HEK-293 Cas9 cells, DNA DSBs at intended sites were detected using T7E1 assay and Sanger sequencing, and the expression of p53 and RFP proteins was significantly disrupted. In A549 CRISPRi cells, gRNAs targeting p53 and SETD9 promoter regions repressed p53 and SETD9 gene transcription approximately 75% and 65%, respectively. In p53 gRNA expressing virus infected A549 CRISPRi single cell clones, approximately 95% p53 transcription repression was detected. Taken together, our data suggest that these CRISPR Tool cell lines are valuable tools that greatly simplify the study of human gene function and provide potential applications for precise gene knockout and knockdown in human cells.

P363/B371

Digestive Enzyme Genes of a Sundew, *Drosera Adelae*: Mechanisms Underlying the Adhesive Trap-specific Gene Expression.

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Carnivorous plants have adapted themselves to the nutrient-deficient habitats. They can obtain nitrogen and phosphate, which are necessary for amino acid and nucleotide syntheses, from insects. *D. Adelae* is a carnivorous plant with an adhesive trap, which secretes viscous digestive fluid from the glandular tentacles on the surface of the leaves. In the digestive fluid, there are many proteins that are induced by wounding and pathogen attack in ordinary plants. Therefore, *D. Adelae* is thought to make use of the defense-related proteins for self-defense and carnivory (Okabe *et al.*, 2005). Recently, we explored expression mechanisms of digestive enzymes in the fluid and suggested that the S-like RNase gene *da-I* is regulated by glandular cell-specific demethylation of its promoter (Nishimura *et al.*, 2013, 2014).In the current study, focusing on five digestive enzyme genes, *Cysp1*, *Glu1*, *Hel1*, *Chi1* and *Tlp1*, we examined the relationship between their expression and promoter methylation. The result showed that most of these genes are exclusively expressed in a glandular cell-specific manner, but methylation profiles differ among these genes. For *Cysp1* and *Glu1*, the promoters were unmethylated only in glandular cells like *da-I*. However, for *Chi1* and *Tlp1*, the promoters were unmethylated in all organs. Interestingly, the gene encoding the DNA demethylase DEMETER, which induces genome-wide demethylation in endosperm in Arabidopsis and rice (Kawashima and Berger, 2014), was also highly expressed in the glandular cells of *D. Adelae*. Accordingly, it seems that *D. Adelae* makes some of the defense-related genes be constitutively expressed via unmethylation in a glandular cells-specific manner for carnivory.

P364/B372

Genomic an notation of 45,600 Bp Region of 3l Chromosome in D. *Takahashii*.

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Drosophila melanogaster, the common fruit fly, is one of the most versatile model organisms in biology, used for a wide range of research centered around cellular mechanisms of complex eukaryotes. In this project we use the D. *melanogaster* genome as a reference to identify and annotate coding regions in Drosophila *takahashii*. This project is part of a collaborative effort of the Genomics Education

Partnership (GEP). Here we use conservation-based analysis and various online tools such as the Drosophila melanogaster genome browser, Flybase, Gene Model Checker and Gene Record Finder to analyze a 45,600 base pair segment of chromosome 3L in *D. takahashii* labeled contig 42. The BLAST of D. *melanogaster* indicates the presence of genes ADP-ribosylation factor GTPase activating protein 3 (ArfGAP3), Maelstrom (mael), lethal(3)04053 (l(3)04053), CG32454, CG14451, CG11367, CG32452, CG14450, CG8745, Aminotransferase class-III (Oat) and CG11241 genes in the contig. The analysis of the *D. takahashii* sequence supports the BLAST results for the aforementioned genes except for CG8745 and Oat which are found in a different region in D. *melonogaste*r. Discrepancy in exon number and placement was resolved using the small exon finder and protein BLAST analysis to select the most conserved model. The results and the data collected from this research will be submitted for review and inclusion to the GEP data repository.

P365/B373

Transcriptomic an alysis of Novel Mycobaacteriophage Herbertwm.

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The genus *Mycobacterium* contains over 190 species and is responsible for significant morbidity and mortality in humans and other animals. In addition to well-studied human pathogens such as *Mycobacterium tuberculosis, leprae,* and*abscessus*, the genus includes many non-tuberculosis environmental species that dwell in the soil and water supply, species that may serve as useful models for the pathogenic species. With the recently reported successes in phage therapy, there has been an increased interest in expanding the diversity of phages that infect hosts within the *Mycobacterium* genus. The goal of this project was to identify phages that infected *Mycobacterium aichiense,* a host for which there are currently no reported bacteriophages. Using standard methods to isolate phages from soil samples, we isolated approximately 50 phages infecting this host from local soil samples. All of the phages had consistently low titers and required optimization of DNA isolation techniques. Ten isolates were sequenced. In each case, sequencing resulted in a ~50 kb phage that may represent a new subcluster within the a cluster of mycobacterial phages; this sequence corresponds to a prophage in the *M. Achiense* genome that we called Herbert WM. Surprisingly, in each sample, there was also a 11 kb "phagelet" that shared little identity with the 50 kb HerbertWM prophage. The phagelet encodes 20 genes including a terminase, a serine-threonine kinase, a head-to-tail adaptor, a head-to-tail stopper and 16 hypothetical proteins of unknown function. While all the isolates contained both the prophage and the phagelet, the stoichiometry of the two phages differed among the various samples. In order to investigate this novel "phagelet" further, we performed RNA-Seq analysis at different time points following infection of the host, including: a time point before phage was added to the sample; and then at 0, 5, 15, 30, 60, and 120 minutes following infection. This analysis revealed, somewhat surprisingly, that in addition to expression of the repressor, many prophage genes were expressed at low levels even in the absence of infection with the phagelet. Phagelet genes were expressed as early as t=5 minutes post infection and continued to increase throughout the time periods analyzed with the few structural genes being expressed late in infection. Prophage (Herbert WM) genes did not significantly upregulate until t=60 min. This represents the first instance of satellite-like phage in the mycobacterial genus, one that may provide valuable tools for manipulating and understanding mycobacterial genomes.

P366/B374

Developing Tools to Detect Translation of the 3' Untranslated Regions of Messenger RNAs.

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Upon viral infection multiple host response mechanisms are initiated, such as the activation of Ribonuclease L (RNase L). The canonical role of RNase L is to cleave host and viral mRNAs. However, activated RNase L is also known to bind to ribosome termination/recycling factors ATP Binding Cassette subfamily E member 1 (ABCE1) and Eukaryotic Release Factor 3 (eRF3) indicating its potential role in ribosome recycling and termination. When ribosome recycling is impaired, ribosomes can bypass the canonical stop codon and translate the 3' Untranslated Region (3'UTR) of mRNAs. Prior ribosome profiling data from our lab suggests that RNase L interferes with ribosome recycling, and potentially triggers 3'UTR translation. Here we have developed 3'UTR reporters to investigate 3'UTR translation in RNase-L activated cells and detect myc-tagged 3'UTR peptides by Western Blot. We observe translation events within the 3'UTRs of non-activated cells, establishing the utility of these reporters. Future studies may investigate changes in 3'UTR translation within RNase-L activated cells.

P367/B375

The Complete Chloroplast Genome of *Lagenaria Siceraria* (mol.) Standl.

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Lagenaria siceraria (Mol.) Standl. commonly known as calabash plant in West Africa, is a highly utilized aesthetic crop because it exhibits the widest variations in fruit shape. However, the phenotypic variation of the plant's fruit shape is continuous and difficult to quantify, with polymorphic and numerous landraces exhibiting diversity in fruit shapes. To decipher these variation, a study on the complete chloroplast genome of the species was carried out. A total of 44 sequences of *L. siceraria* stored in the National Center for Biotechnology Information (NCBI) genebank was extracted and annotated using GeSeq program in CHLOROBOX. The organellar genome map of the species is a circular molecule of about 157,145 base pairs. The chloroplast genome has two inverted repeats regions a and B (IR_A and IR_B) of 25,910bp that contain the rRNA genes and a few other duplicated genes. These repeats are seperated by two single-copy sequences, one small and one large (of 8,735bp and 17,974bp respectively) that contain the bulk of the tRNA and protein-coding genes, with an overall Guanine-Cytosine content of 37.7%. There are a total of 131 genes in the genome, including 86 protein-coding genes, 37 tRNAs genes and 8 rRNAs genes. Also, the intergenic and untranscribed region such as photosystem II and tRNA genes were highly variable in the chloroplast genome of *Lagenaria siceraria*., All aligned sequences showed that *L. siceraria* chloroplast genome possesses high sequence similarity, which is an indication that it is conservative, although it possess some region of divergence. Coding regions has been recorded to be more conserved than the non-coding regions; hence the ribulose bisphosphate carboxylase gene (rbcL) and Acetyl-coenzymeA carboxylase gene (accD) found in the species are coding regions which has been reported to be divergent in plastome which may be linked to the variations in the fruit shape of the crop. This study therefore holds potential in the improvement of L*. siceraria*, which will enhance the utilization and demand of the crop in sub-Sahara region. Keywords: Chloroplast, Genome, Gene expression, *Lagenaria siceraria* (Mol.) Standl.

P368/B376

Transcription Factor Foxa1 Clusters Dna through Wetting-mediated Capillary Forces.

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Direct interactions between enhancers and promoters are necessary for the activation of gene regulatory programs in vivo. However, how transcription factors, co-factors, and general transcriptional machinery work in concert to physically bring DNA together in space and time remains unclear. Here, we used super-resolution TIRF to image the interactions of single-molecules of linearized lambda-phage DNA and Forkhead Box Protein A1 (FoxA1). We discovered that FoxA1 wets DNA, generating capillary forces that reel-in DNA into clusters on the order of 20 KB in a DNA tension-dependent manner. To understand the source of this reeling-in force, we imaged pairs of DNA molecules that were bound spatially proximal to each other, and we found that these DNA molecules exhibited “zipping”, consistent with a mechanism where FoxA1 minimises its surface area due to surface tension effects, and, consequently, generates capillary forces. We proposed that the origin of these capillary forces is a combination of FoxA1 self-interactions and FoxA1-DNA adhesion. To test this theory, we generated a C-terminal truncation of FoxA1, a disordered region that we thought may play a role in FoxA1 self-interaction. FoxA1ΔCTD did not cluster DNA, suggesting that FoxA1-FoxA1 interactions are relevant for capillary force generation. This reveals that transcription factors could display a range of behaviors depending on their structure. To investigate this, we used Tata-Box Binding protein (TBP), a general transcription factor, and linker histone H1, a chromosomal architecture protein structurally similar to FoxA1. We found that TBP wets DNA, forming droplets that translocate along the strand, though was unable to reel-in DNA. However, H1 reeled-in DNA with stronger capillary forces, consistent with its known compacting activity. We propose that capillary-force-induced DNA clustering is a novel mechanism that may play a fundamental role in DNA organization in the cell nucleus.

RNA Localization and Transport

P369/B377

Localized RNA Translation as a Modifier of Protein Properties and Function.

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The localization of RNA transcripts to particular subcellular compartments is a mechanism used to localize numerous transcripts in polarized cells. RNA localization plays a critical role in several processes, including cell migration, synaptic plasticity and embryonic axis specification, and is deregulated in cancer metastasis and many neurological diseases. In most cases, localized RNAs are believed to be locally translated, which can lead to an increase in protein concentration at these locations. However, recent evidence has indicated that translation can have additional consequences on the function of the encoded proteins. Some potential mechanisms include interactions between nascent proteins and RNA-bound factors, which can direct the released protein to distinct fates, or the co-translational assembly of proteins into complexes. This study investigates the hypothesis that RNA localization affects the encoded protein’s function by restricting translation to specific subcellular regions. To test this hypothesis, we use as a model the localized Net1 RNA, which is concentrated at peripheral protrusions and encodes a Rho family GTPase activator. We have developed a method that allows us to specifically mislocalize the Net1 RNA from protrusions and force it to adopt a perinuclear distribution. Interestingly,

when the Net1 transcript is mislocalized in MDA-MB-231 cells, RhoA signaling is decreased concomitant with a reduction in cell migration speed and invasion. These results are consistent with the model that local production of Net1 affects the function of the encoded protein, and thus alters the regulation of downstream cellular physiological processes.

P370/B378

Mitochondrial Volume Fraction Controls Translation of Nuclear-encoded Mitochondrial Proteins.

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Mitochondria are dynamic in their size and morphology, yet must also precisely control their protein composition according to cellular energy demand. We have found that cells are able to use mitochondrial morphology to post-transcriptionally coordinate protein expression with metabolic demands through enhanced mRNA localization to the mitochondria. As yeast switch to respiratory metabolism they increase their mitochondrial volume fraction, this drives the localization of nuclear-encoded mitochondrial mRNAs to the surface of the mitochondria. Through artificial tethering experiments, we show that this mitochondrial localization is necessary and sufficient to increase protein production to levels required during respiratory growth. Furthermore, we find that this mRNA sensitivity to mitochondrial volume fraction is driven by the speed of translation of the ORF. This points to a mechanism by which organelle volume fraction provides feedback to regulate organelle-specific gene expression through mRNA localization while potentially circumventing the need to directly coordinate with the nuclear genome.

P371/B379

RNAs Are Transported on Microtubules and Localized to the Sarcomere in Skeletal Muscle.

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RNA localization and local translation of proteins is a critical mechanism by which cells specialize subcellular compartments. Specialized cells with complex cyto-architecture, such as neurons and muscle, have evolved complex mechanisms to ensure proper localization of RNA and local translation of encoded proteins in diverse regions of the cytoplasm. Muscle cells face unique challenges to appropriately distribute RNA. They are large syncytia of thousands of nuclei containing in their common cytoplasm an intricate, force-generating cytoskeletal apparatus that restricts diffusion of gene products. Despite literature describing localized RNAs in muscle, the majority of work in the RNA localization field has focused on neurons. As such, the mechanisms, trans factors, and cis elements involved in localizing RNAs and translational activity in skeletal muscle are poorly understood. In this study, we characterize the localization of known and novel localized RNAs in mouse muscle and assess RNA transport and translation regulation in live muscle cells. We imaged single molecules of RNA in *in vivo* skeletal muscle, finding that many are associated with components of the muscle sarcomere and that the microtubule cytoskeleton is required for efficient RNA distribution from the myo-nuclei. We then used live-cell RNA and nascent peptide tracking in cultured myotubes to interrogate the mechanistic underpinnings of observed in *vivo* localization patterns for target RNAs. We find that translating and non-translating mRNAs are actively transported and associate with the sarcomere in an in *vitro* muscle development paradigm. We additionally sought to characterize the full sarcomere-localized transcriptome. To this end, we utilized a proximity biotinylation approach to label sarcomere Z-lines in myotubes in *vitro*. We

are adapting this method to isolate sarcomere RNAs from in *vivo* muscle and will use RNAseq to identify the full repertoire of sarcomere localized transcripts. This dataset will power bioinformatics analyses that will identify candidate localization-directing cis elements for further evaluation. The results of these efforts will increase our understanding of post-transcriptional gene regulation in basic biology, as well as clarifying the role of RNA localization and local translation in the assembly and maintenance of the muscle sarcomere.

Nuclear Envelope 1

P2641/B380

The Polo-like Kinase (PLK-1) Merges the Parental Genome Into A Single Nucleus by Promoting Lamina Disassembly during Mitosis In *C. Elegans* Embryos. G. Velez Aguilera[1], S. Nkombo Nkoula[1], L. Van Hove[1], D. Paouneskou[2], J. Link[2], B. Ossareh-Nazari[1], N. Joly[1], V. Jantsch[2], L. Pintard[1];
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After fertilization, the haploid gametes of egg and sperm are replicated into separate pronuclei surrounded by a nuclear envelope but before the first zygotic division, the nuclear envelopes disassemble, allowing both sets of chromosomes to be incorporated into a single nucleus in daughter cells after mitosis. Here we show that the polo-like PLK-1 phosphorylates the single C. elegans lamin LMN-1 to promote lamina disassembly during mitosis and to merge the parental chromosomes into a single nucleus. We demonstrate that expression of non-phosphorylatable versions of LMN-1 prevents Lamina depolymerization during mitosis, which is sufficient to induce the formation of embryos with a paired nuclei phenotype, containing either the maternal or paternal chromosomes, in each daughter cell at the two-cell stage. Finally, we reconstitute in vitro the assembly of lamin filaments and their depolymerization by PLK-1 and demonstrate the importance of LMN-1 phosphorylation sites in this process. Our findings indicate that PLK-1 is a key regulator of the lamin dynamics in C. elegans and possibly also in other organisms.

P372/B381

Effect of Hiv-1 Infection on the Distribution of the Transmembrane Nucleoporin Pom121.
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Over the last decade it became evident that nucleoporins are integrally linked to the life cycle of the HIV-1 genome. Recently an N-terminal truncated form of the nucleoporin POM121C was reported to inhibit HIV-1 replication[1] and earlier it was shown that the knockdown of POM121 inhibited HIV-1 replication at the nuclear import step[2]. Our group had data from a HeLa cell line with an integrated HIV-1 test genome, which carried the BGlg RNA aptamer binding site[3], that showed how POM121 (labeled with tdTomato) relocates within the nuclear envelope and that a sub-fraction of POM121 colocalizes with the viral full-length RNA. Conducting multi-channel imaging experiments with our home-built state of the art microscope, this time using Jurkat, and primary human T-cells infected with pNL4-3-GFP HIV-1 virus (NIH AIDS reagent 11100), we observed progressive changes in the distribution of POM121. Starting from its normal abundance within well-spaced nuclear pores at time zero, POM121 separated into aggregates later in the infection cycle. We show that cells showing this phenotype are

not apoptotic using an an nexin V-Alexa647 co-stain[4] (Biolegend 640912). Our data point towards a new mechanism for at least a sub-class of HIV-1 full length genomes during cell nuclear export that provide further insight into how the HIV virus manipulates the host cells transport machinery, possibly to arm itself for the next round of infection. [1] Saito, H., Takeuchi, H., Masuda, T., Noda, T., Yamaoka, S., 2017. N-terminally truncated POM121C inhibits HIV-1 replication. PLoS One 12, e0182434. doi: 10.1371/journal.pone.0182434. eCollection 2017. PubMed PMID: 28873410; PubMed Central PMCID: PMC5584925. [2] Guo J, Liu X, Wu C, Hu J, Peng K, Wu L, Xiong S, Dong C. The transmembrane nucleoporin Pom121 ensures efficient HIV-1 pre-integration complex nuclear import.Virology. 2018 Aug;521:169-174. doi: 10.1016/j.virol.2018.06.008. Epub 2018 Jun 25. PubMed PMID: 29957337; PubMed Central PMCID: PMC6309762. [3] Chen J, Nikolaitchik O, Singh J, Wright A, Bencsics CE, Coffin JM, Ni N, Lockett S, Pathak VK, Hu WS. High efficiency of HIV-1 genomic RNA packaging and heterozygote formation revealed by single virion analysis. Proc Natl Acad Sci U S A. 2009 Aug 11;106(32):13535-40. doi: 10.1073/pnas.0906822106. Epub 2009 Jul 23. PubMed PMID: 19628694; PubMed Central PMCID: PMC2714765. [4] Zaitseva E, Zaitsev E, Melikov K, Arakelyan A, Marin M, Villasmil R, Margolis LB, Melikyan GB, Chernomordik LV. Fusion Stage of HIV-1 Entry Depends on Virus-Induced Cell Surface Exposure of Phosphatidylserine. Cell Host Microbe. 2017 Jul 12;22(1):99-110.e7. doi: 10.1016/j.chom.2017.06.012. PubMed PMID: 28704658; PubMed Central PMCID: PMC5558241.

P373/B382

Torsin Regulation of Lipid Metabolism in Nuclear Membrane Remodeling and Disease.

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Torsins are atypical AAA+ proteins of the endoplasmic reticulum (ER) and nuclear envelope (NE). Their loss causes vesicle-like structures to accumulate in the NE which label for some nucleoporins. The human torsinA gene is also responsible for at least two different neurological diseases: 1) a recessive congenital syndrome and 2) a dominantly-inherited dystonia. We previously showed that fly *dTorsin* regulates the lipid metabolic enzyme, LIPIN, that converts phosphatidic acid (PtdA) into diacylglycerol (DAG) (Grillet, *et al*, Dev Cell (2016). Now, via a mini screen, we genetically define a pathway that functionally links torsin to LIPIN. This includes that the nuclear membrane defects of torsin loss are rescued by RNAi against these genes, while gene overexpression induces their appearance. We also examined the disease relevance of LIPIN dysregulation. This identified excess LIPIN-mediated PtdA conversion to DAG in 1) dystonia patient iPSC-derived neurons, 2) the brains of dominant TorsinA-dystonia mice and 3) three mouse models of recessive TorsinA disease. Further, using *Lipin1* gene knock-out mice, we demonstrated that LIPIN dysregulation contributes to neurodegeneration, abnormal motor behaviors, and poor survival of torsinA disease mice. In conclusion, these data show that torsin loss affects the cell via a pathway that controls PtdA to DAG metabolism. This includes the classic nuclear membrane defects of torsin loss, as well as disease-like pathology and symptoms in torsinA disease model mice.

P376/B385

TorsinA Is a Multi-tool AAA+ ATPase within the Nuclear Envelope That Acts through Distinct Functional Assembly States.

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Mutations in the *TOR1A*gene, which encodes the nuclear envelope-localized AAA+ ATPase torsinA, cause a spectrum of poorly understood neurological diseases including DYT1 dystonia and severe arthrogryposis. While torsinA function is required for nuclear-cytoskeletal coupling and nuclear pore complex (NPC) biogenesis, the molecular mechanism(s) underlying how torsinA mediates these functions and their relationship to human*TOR1A*disease pathogenesis remain unclear. Here, we determined the functional assembly state of torsinA in the nuclear envelope of living cells using fluorescence fluctuation spectroscopy. Because AAA+ proteins generally function as homo-hexameric molecular chaperones that structurally remodel their substrates, we expected to observe homo-hexamers of torsinA in the nuclear envelope. In contrast, the in *vivo*homo-oligomerization of torsinA was limited to a homo-trimer. Unexpectedly, the deletion of its membrane-associating N-terminal domain (NTD) resulted in the assembly of torsinA homo-oligomers larger than expected for a homo-hexamer. By measuring the assembly states of constructs containing torsinA monomers that were covalently linked into artificial dimers, trimers, tetramers, pentamers, and hexamers, we revealed that torsinA polymerizes following the rate limiting step of dimerization at discreet sites within the nuclear envelope. Next, we investigated the physiological relevance of torsinA polymerization by testing its role during nuclear-cytoskeletal coupling and NPC biogenesis. Interestingly, we found that torsinA polymerization was required for nuclear-cytoskeletal coupling but dispensable for NPC biogenesis. Moreover, we determined that membrane-association was critical for NPC biogenesis but detrimental to nuclear-cytoskeletal coupling. In fact, nuclear-cytoskeletal coupling was prevented when the NTD was converted into a transmembrane domain or when a previously described proteolytic cleavage event that selectively removes the NTD was inhibited. Finally, we showed that the ability of torsinA to hydrolyze ATP was not important for NPC biogenesis, whereas it was essential for nuclear-cytoskeletal coupling. Taken together, our results suggest that torsinA is a multi-tool AAA+ protein that acts via different functional assembly states to execute distinct functions within the nuclear envelope. We propose that torsinA functions as a soluble polymeric chaperone to promote proper nuclear-cytoskeletal coupling, whereas it mediates NPC biogenesis as a lower-order homo-oligomer by potentially remodeling the inner nuclear membrane.

P374/B383

Cytoplasmic Capes in the Nuclear Envelope Are Closely Associated with Non-Canonical RNA Export and Receive Proteins from the Endosome System.

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It is increasingly clear that nuclear-cytoplasmic communication is not limited to nuclear pores, with both proteins and RNA having alternative routes between the cytoplasm and nucleoplasm. We have previously characterized large invaginations of the nuclear envelope in the *Drosophila* larval salivary gland, known as ‘cytoplasmic capes’, which are enriched for the immature, membrane-bound EGF receptor ligand mSpitz and encapsulate endosome-related organelles containing ubiquitylated proteins.

Closely associated with these structures are groups of perinuclear vesicles strongly resembling those seen at sites of mega-RNP export *via* a budding mechanism. Here, through knockdown of key endosomal regulators, we demonstrate that mSpitz delivery to capes requires passage through the endosomal system despite the immediate connection between the nuclear envelope and the ER. Using an improved in *situ* hybridization technique, we also show that capes are closely associated with sites of RNP export as well as the dFrizzled2 receptor C terminal fragment, a core component of this export pathway. Video microscopy of glands in intact larvae indicates that cytoplasmic capes are stable structures that persist for at least 90 minutes without conspicuous growth. We further show that cytoplasmic capes appear with the growth of the salivary gland rather than its developmental stage. Finally, we show that the large F-actin binding protein βH-spectrin, which modulates endosomal trafficking, as well as its partner α-spectrin are required for cape formation. Cytoplasmic capes therefore represent an interesting subspecialization of the nuclear envelope where endosomal trafficking and RNP export are closely associated.

P375/B384

Mechanism of Nucleotide Binding to TorsinA.

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The loss of nuclear envelope (NE) integrity leads to several diseases including muscular dystrophy, cardiomyopathy, dystonia, and cancer. Dysfunction in NE protein homeostasis results in changes to NE structure and may drive this loss, though the mechanistic basis for maintaining NE protein folding and assembly remains unclear. TorsinA (TorA) belongs to the AAA+ (ATPases Associated with various cellular Activities) family of protein unfoldases, which couple the chemical energy from nucleotide hydrolysis to mechanical work used in protein unfolding and quality control. Several studies demonstrate a role for TorA in regulating NE protein assembly though a mechanistic basis understanding of how TorA couples its ATPase activity to its cellular function is unclear. We have measured steady state ATPase activity of full-length TorA in the presence and absence of its cofactor LAP1 in *vitro*. We find that TorA does not need LAP1 to bind and hydrolyze ATP, in contrast to previous studies. In addition, the TorA-LAP1 interaction does not increase TorA ATPase activity at saturating ATP concentrations, but the interaction increases TorA ATP binding affinity. Our data define a novel role for LAP1 in regulating TorA ATPase activity and provide a framework for understanding how this enigmatic enzyme couples nucleotide binding and hydrolysis to mechanical work.

P377/B386

The Sun1 Splicing Variants Sun1_888 and Sun1_916 Differentially Regulate Nucleolar Structure.

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The nucleolar structure is highly dynamic and strictly regulated in response to internal cues, such as metabolic rates, as well as to external cues, such as mechanical forces applied to cells. Although the multi-layered nucleolar structure is largely determined by the liquid-like properties of RNA and proteins, the mechanisms regulating the morphology and number of nucleoli remain elusive. The linker of the nucleoskeleton and cytoskeleton (LINC) complex comprises inner nuclear membrane Sad1/UNC-84 (SUN) proteins and outer nuclear membrane-localized nesprins. We previously showed that depletion of SUN1 proteins affects nucleolar morphologies. This study focuses on the function of SUN1 splicing variants in determining nucleolar morphology because the functional differences among SUN1 splicing

variants remain unknown. An RNA interference strategy showed that the dominantly expressed variants, SUN1_888 and SUN1_916, were crucial for nucleolar morphology but functionally distinct. In contrast, depletion of nesprin-1 or nesprin-2 did not affect nucleolar morphology or numbers. In addition, depletion of either SUN1_888 or SUN1_916 altered the chromatin structure and affected the distribution of histone modifications. Based on these results, we propose a model in which the LINC complex plays a role in modulating nucleolar morphology and numbers via chromatin.

P378/B387

Destabilization of the Linc Complex Alters Spindle Orientation and Apico-basal Polarity in Glandular Epithelia.

V. Narayanan[1], Q. Zhang[2], T. Lele[2], D. Conway[1]; [1]Virginia Commonwealth University, Richmond, VA, [2]University of Florida, Gainesville, FL.

Destabilization of the LINC Complex Alters Spindle Orientation and Apico-basal Polarity in Glandular Epithelia Vani Narayanan[1], Qiao Zhang[2], Tanmay P. Lele[2], Daniel E. Conway[1] [1]Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, VA, 23284 [2]Department of Chemical Engineering, University of Florida, Gainesville, FL 32611 the nuclear LINC (linker of the nucleoskeleton and cytoskeleton) complex, that serves as a bridge between the nuclear lamina and cytoskeleton, has recently emerged as another critical structure and mechanosensitive region of the cell. Mutations associated with the LINC complex components has its implications in cancer, but it remains unclear how cell-level changes translate into tissue-level malignancy. We hypothesized that decoupling the nucleus from the cytoskeleton would affect formation and homeostasis of the epithelium. To investigate the role of the LINC complex in epithelial cells, we developed an MDCK II cell line expressing inducible dominant negative (DN) KASH, a peptide that disrupts the endogenous nesprin-SUN interactions and blocks nuclear-cytoskeleton interactions. Phenol-free reduced growth factor reduced Matrigel™ (Corning) was used for all 3D cell-culture experiments. (DN) KASH induced acini exhibited asymmetric cell division over a period of 24 hours resulting in disrupted lumen morphology. Additionally, we observed that decoupling the nucleus from the cytoskeleton affected mitotic spindle orientation in 2D monolayers grown on transwell permeable supports and resulted in stratification when allowed to grow over an extended period of time. To investigate whether polarity disruption followed or preceded asymmetric cell division as a result of LINC disruption, the (DN) KASH induced acini were observed at varying time points post induction. An increase in altered polarity was observed over a period of 48, 72 hours and 7 days, respectively. To elucidate cellular proliferation, disruption in polarity and cell survival in the luminal space, LINC disrupted acini were studied for β1-integrin expression. Acinar lumenal filling was accompanied with increased expression of β1-integrin confirming altered polarity with LINC complex disruption and suggesting the possibility of an over-compensatory mechanism involving focal adhesions. Furthermore, blocking proliferation via aphidicolin (a reversible inhibitor of nuclear DNA replication) prevents lumen occlusion, indicating that cell division is required for acinar lumen collapse. Taken together, our results indicate that nucleo-cytoskeletal coupling is necessary for cells to sense, respond to mechanical cues and maintain normal cellular function on a tissue scale.

P379/B388

Determining the Regulatory Role of LINC Complex in Smad Signaling and in Expression of Inner Nuclear Membrane Protein MAN1 in the Presence of TGF-β.

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Transforming growth factor (TGF)-β plays a central role in health and disease. The TGF-β pathway acts through Smads, a family of transcription factors that are activated by phosphorylation in the cytoplasm and translocate into the nucleus to modulate gene expression. Post-translational modifications, trafficking and functional regulation of Smads has been the subject of numerous studies. For instance, it has been shown that MAN1, an inner nuclear membrane specific protein, negatively regulates Smad-dependent TGF-β signaling. Our recent work demonstrated that SUN2, a component of the linker of nucleoskeleton and cytoskeleton (LINC) complex, influences Smad localization and gene regulation, suggesting that mechanical signals transmitted by LINC might also influence Smad signaling. Despite these studies, however, it remains to be elucidated how, in the presence of TGF-β, nuclear envelope (NE)-associated proteins and the LINC complex act together to regulate Smad signaling. In this study we provide new insight into how, in the presence of TGF-β1, the LINC complex affects localization of activated Smads (phosphoSmad2/3 and Smad4), the NE localization and total expression level of MAN1, and the expression of Smad target genes. Here we take advantage of primary wild types (WTs) and *Sun2*$^{-/-}$ mouse embryonic fibroblasts (MEFs). Cells, plated in conditions to engage their integrins (fibronectin-coated surfaces), were treated with TGF-β1 (10 ng/ml) for 45 minutes. Fluorescent microscopy demonstrates that, in response to TGF-β1, the nuclear translocation of activated Smads occurs in both the presence and absence of SUN2. However, in contrast to WTs, *Sun2*$^{-/-}$ MEFs show higher levels of nuclear phosphoSmad2/3 and Smad4 even in the absence of TGF-β1. We also observe an increase in the pool of MAN1 at the NE in both WTs and *Sun2*$^{-/-}$ MEFs after TGF-β1 stimulation, but this is more profound in *Sun2*$^{-/-}$ MEFs. Western blot analysis of MAN1 confirms that, in response to TGF-β1, expression of MAN1 increases in *Sun2*$^{-/-}$ MEFs compared to WT MEFs. Although the effect of these changes on gene expression in these model cell lines is still under investigation, our previous work suggests that Smads inefficiently regulate their target genes in *Sun2*$^{-/-}$ cells despite the elevated levels of nuclear Smads. Our results show that SUN2 plays a critical role in the expression and localization of MAN1, an established negative regulator of TGF-β signaling. Our data also suggest that, in addition to soluble factors, mechanotransduction through LINC complex influences the nuclear aspects of TGF-β and Smad signaling, likely through regulation of MAN1.

P380/B389

The Giant KASH Protein ANC-1 Functions with and Without LINC Complexes to Position Nuclei and Other Organelles.

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Nesprin-1 and 2 are outer nuclear membrane KASH proteins that interact with SUN proteins to form Linker of nucleoskeleton and cytoskeleton (LINC) complexes to position nuclei. LINC is important for many cellular and developmental processes; defects in LINC lead to disease. We use the *C. elegans* hypodermis as a model because it contains 139 syncytial nuclei that are normally evenly spaced apart. In *C. elegans*, ANC-1 is an ortholog of Nesprin 1 and 2 that is over 8500 residues with a conserved N-terminal calponin homology (CH) domain that binds actin and a C-terminal KASH domain that interacts with SUN protein UNC-84. Mutations in *anc-1* or *unc-84* disrupt nuclear anchorage. In our working model, ANC-1 tethers nuclei to the actin cytoskeleton. However, our new genetic data do not fit with the model. First, *anc-1(null)* mutants have much more severe nuclear anchorage defects than *unc-84(null)* or *anc-1(Δkash)* mutants, suggesting an unknown, LINC-independent role of ANC-1. Second, CRISPR deletion of the N-terminal CH domains had no nuclear anchorage defects, indicating that a

shorter isoform, *anc-1b*, without the CH domain is sufficient to position nuclei in the hyp7. In support of this, early stop codons and RNAi targeted against the first 1500 residues of *anc-1* do not cause nuclear anchorage defects and we isolated a cDNA representing *anc-1b*. Interestingly, CRISPR-mediated deletion of the large repeats in the middle of ANC-1 or the N-terminal residues of ANC-1b caused severe nuclear anchorage defects, implicating an essential role for the cytoplasmic domains of ANC-1 in nuclear positioning. Using CRISPR editing, we show that GFP::ANC-1b localizes to the nuclear envelope and what appears to be the ER. In addition, mitochondria and lipid droplets are also mislocalized in *anc-1(null)* mutants. We hypothesize that the cytoplasmic domains of ANC-1 regulate the global cytoskeleton and *anc-1* mutants disrupt the positioning of nuclei, mitochondria, lipid droplets, and other organelles. Finally, deletion of both the transmembrane domain and the KASH domain caused significantly more severe nuclear anchorage defects compared with *anc-1(Δkash)*, indicating that targeting ANC-1 to membranes is important for its LINC-independent function. In summary, we propose a new role for the giant KASH protein ANC-1 in organizing the cytoplasm to position multiple organelles.

P381/B390

How FHOD1 Interacts with Nesprins to Mediate Nuclear Movementhow Fhod1 Interacts with Nesprins to Mediate Nuclear Movementhow Fhod1 Interacts with Nesprins to Mediate Nuclear Movementhow Fhod1 Interacts with Nesprins to Mediate Nuclear Movement.

R. S. M. Lim, V. E. Cruz, **T. U. Schwartz**; Massachusetts Institute of Technology, Cambridge, MA.

Linker of nucleoskeleton and cytoskeleton (LINC) complexes form bridges across the nuclear envelope to connect nucleoskeleton and cytoskeleton for force transmission. LINC complexes are composed of KASH proteins traversing the outer nuclear membrane, and SUN proteins crossing the inner nuclear membrane. While the SUN-KASH interaction is structurally understood in principle, neither tethering to the cytoskeleton nor the nucleoskeleton is resolved. Here we present the crystal structure of FHOD1 interacting with a specific spectrin repeat within Nesprin2. The structure reveals a novel type of engagement, including a highly conserved charge network. We were able to use the structural information to find at least one additional spectrin repeat that can also interact with FHOD1. With this data we can start to unravel the details of the engagement of the LINC complex with the actin cytoskeleton.

P382/B391

The KASH Protein UNC-83 Serves as a Cargo Adapter at the Nuclear Envelope for Microtubule Motors and Activates Kinesin-1.

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Many intracellular cargos move along microtubules using a combination of kinesin-1 and dynein motors. The *C. elegans* KASH protein UNC-83 is targeted to the outer nuclear membrane, where it serves as a cargo adapter to recruit both kinesin-1 and dynein motors to nuclei. Nuclei migrate towards the plus ends of microtubules using primarily kinesin-1 motors in *C. elegans* embryonic hyp7 cells. However, in *C. elegans* larval P cells, nuclei move toward the minus ends of microtubules, favoring dynein. It is unknown how nuclei regulate the decision between using kinesin-1 or dynein motors to move nuclei in opposite directions at different times in development. In our model, different isoforms of UNC-83, alternatively expressed in hyp7 or P cells, favor kinesin or dynein, respectively. Specifically, our previous

genetic data suggest that a short *unc-83c* isoform is sufficient for kinesin-driven nuclear migration in hyp7 cells, but a longer *unc-83a/b* isoform is required for dynein-driven movements in P cells. We hypothesize the short *unc-83c* isoform activates kinesin-1 and/or inhibits dynein, while the longer *unc-83a/b* isoform inhibits kinesin-1 and/or activates dynein. We are using a combination of in *vivo* developmental genetics and in *vitro* microtubule motor assays to test our model. Purified full-length kinesin-1/KLC-2 tetramers have no motor activity in our TIRF assays. However, the addition of the cytoplasmic domain of UNC-83c robustly activates kinesin-1 in our microtubule motor assays. To test the role of UNC-83c in *vivo,* we engineered a mutation using CRISPR/Cas9 into the EWD motif of UNC-83. EWD motifs are thought to mediate interactions with kinesin light chains. *unc-83(EWD-GSA)* mutant animals have a severe hyp7 nuclear migration defect, suggesting they are not capable of activating kinesin-1. We are currently using CRISPR/Cas9 genome editing to express *unc-83* isoforms in reciprocal tissues. We are deleting domains unique to *unc-83a/b in* P cells and adding the *unc-83a/b* unique regions under control of the *unc-83c* reporter in hyp7 cells. Our combination of in *vitro and* in *vivo* approaches is expected to shed light on the molecular mechanisms of how microtubule motors of opposite directionality are regulated on a single cargo.

P383/B392

***Drosophila* Emerins Control Linc Complex Localization and Transcription to Regulate Myonuclear Position.**

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Mispositioned nuclei are a hallmark of skeletal muscle disease. Although mispositioned nuclei have long been a recognized marker of ongoing muscle repair, recent data suggests a more complicated relationship. Sets of proteins have evolved, and are conserved throughout eukaryotes, to facilitate the transmission of force between the cytoplasm and the nucleus and enable nuclear movement. Intriguingly, many of the genes that encode these proteins are mutated in patients with the muscle disease Emery-Dreifuss muscular dystrophy (EDMD), suggesting that disruptions in nuclear movement and position may contribute to disease progression. Furthermore, the genes mutated in patients with EDMD regulate nuclear movement in various cell types suggesting that their function during nuclear movement has been conserved. Yet how these genes are coordinated to move nuclei is not known. Here we demonstrate that the disruption of EDMD-linked genes caused at least two distinct phenotypes. These different phenotypes are based in opposing effects on the amount of the KASH-domain protein Klarsicht that is localized to the nucleus. The levels of Klarsicht in the nuclear envelope are differentially affected by the opposing activities of two separate emerin proteins, Bocksbeutel and Otefin. When Bocksbeutel is disrupted, Klarsicht protein localization is disrupted and nuclei fail to separate from one another. When Otefin is disrupted, Klarsicht transcription is increased and nuclei separate into the center of the muscle fiber. Furthermore, loss of Otefin is able to rescue the nuclear positioning phenotypes caused by loss of Bocksbeutel. These data indicate that Klarsicht levels in the nuclear envelope are a critical determinant of nuclear position. Additionally, these levels are controlled by two opposing emerin activities that are divided between Bocksbeutel and Otefin.

P384/B393

Investigation of Actin Binding Domains of Nesprin.

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Nuclear mechanotransduction is essential in a wide range of biological functions including nuclear movement, nuclear positioning, cell polarization, and chromatin organization. Genetic mutations of KASH family proteins, to which Nesprin 1 belongs cause human diseases including Emery-Dreifuss muscular dystrophy (EDMD). Nesprin 1 is a component of LINC complexes responsible for transferring nucleo-cytoskeletal forces, and recruits actin through its calponin homology domains. Here, we purified and characterized the actin-recruiting calponin homology domains of human Nesprin 1. Circular dichroism spectroscopy wavelength scans confirm that these domains are mostly alpha-helical. The formation of dimers and monomers was confirmed by multi-angle light scattering. The interaction of Nesprin 1 and actin was confirmed through actin co-sedimentation assays. Our data provide mechanistic insights into actin recruitment and bundling though the calponin homology domains of Nesprin 1.

P385/B394

Structural an alysis of Different Linc Complexes Reveals Distinct Binding Modes.

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Linker of nucleoskeleton to cytoskeleton (LINC) complexes are molecular tethers that span the nuclear envelope (NE) and physically connect the nucleus to the cytoskeleton. LINC complexes are composed of KASH proteins traversing the outer nuclear membrane, and SUN proteins crossing the inner nuclear membrane. They mediate mechanical force transmission across the NE in processes such as nuclear anchorage, nuclear migration, and homologous chromosome pairing during meiosis. Humans have several SUN- and KASH-containing proteins, yet it is poorly understood what governs their proper engagement. To investigate this question, we solved high resolution crystal structures of human SUN2 in complex with the KASH-peptides of Nesprin3, Nesprin4, and KASH5. In comparison to the published structures of SUN2-KASH1/2 we observe alternative binding modes for these KASH peptides. While the core interactions between SUN and the C-terminal residues of the KASH peptide are similar in all five complexes, the extended KASH-peptide adopts at least three conformations. The much improved resolution of the new structures allows for a more detailed analysis of other elements critical for KASH interaction, including the KASH-lid and the cation loop region.

P386/B395

The Actin Binding Domain of Nesprin-2G Is Required for Lumen Formation in Mammary Epithelial Acini.

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The linker of nucleoskeleton and cytoskeleton (LINC) complex connects the nucleus to the cytoskeleton and plays key roles in diverse aspects of cell behavior from positioning the nucleus to regulating cytoskeletal dynamics. The LINC complex is comprised of an outer nuclear membrane nesprin and an inner nuclear membrane SUN protein to form a complex in the perinuclear space through the binding of their KASH and SUN domains, respectively. Studies of the LINC complex have established roles for them in mesenchymal cells, but there are few studies examining them in epithelial cells. Previously, we

showed with dominant negative approaches that disrupting the LINC complex in 3D normal breast epithelia acini, prevented both the formation and maintenance of acini with hollow lumens (Zhang, Q. et al., Current Biology, 2019). Here, we used shRNA approaches to identify the specific LINC complexes involved. Knockdown of nesprin-2, SUN1 or SUN2, but not nesprin-1, prevented the formation of acini with hollow lumens, and concomitantly increased proliferation and apoptosis. Therefore, distinct components of the LINC complex are critical in the development and homeostasis of mammary epithelial tissue. To determine the specific cytoskeletal linkages that are involved in lumen formation, we next re-expressed different fragments of nesprin-2G in knockdown cells. While the N-terminal fragment of nesprin-2G bearing F-actin-binding calponin homology domains was sufficient to rescue lumen formation, the C-terminal fragment containing the microtubule-interacting domain failed to do so. These data show that nesprin-2G interaction with F-actin is required for 3D acini formation and a normal balance of proliferation and apoptosis in the acini. As actin anchors the nucleus in other systems, we hypothesize that disrupting nuclear anchorage may be required for normal acini formation.

P387/B396

Nuclear Size Changes Caused by Local Motion of Cell Boundaries Dilate Chromatin and Intranuclear Bodies.

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The mechanisms by which mammalian nuclear shape and size are established in cells, and become abnormal in disease states are not understood. Here, we tracked motile cells that underwent systematic changes in cell morphology as they moved from 1-D to 2-D micro-patterned adhesive domains. Motion of the cell boundaries during cell motility caused a dynamic and systematic change in nuclear volume. Short time scales (~ 1 h) distinguished the dilation of the nucleus from the familiar increase that occurs during the cell cycle. Nuclear volume was systematically different between cells cultured in 3D, 2D and 1-D environments. Dilation of the nuclear volume was accompanied by dilation of chromatin, a decrease in the number of folds in the nuclear lamina, and an increase in nucleolar volume. Treatment of 2D cells with non-muscle myosin-II inhibitors decreased cell volume, and proportionately caused a decrease in nuclear volume. These data suggest that nuclear size changes during cell migration may potentially impact gene expression through the modulation of intra-nuclear structure.

P388/B397

Cytoplasmic Volume and Limiting Nucleoplasmin Scale Nuclear Size during *Xenopus Laevis* Development by Altering Chromatin Organization.

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A basic question in cell biology is how the size of the nucleus is regulated with respect to cell size. During early *Xenopus laevis* embryogenesis, there are dramatic reductions in both cell and nuclear sizes. This reproducible scaling of nuclear size provides a robust system with which to characterize and identify mechanisms of nuclear size regulation. To test if the volume of embryonic cytoplasm is limiting for nuclear growth, we used microfluidic technology to encapsulate gastrula stage *X. laevis* extract and nuclei in droplets of defined volume and shape. We found that cytoplasmic volume and composition, but not cytoplasmic shape, contribute to nuclear size scaling. To identify cytoplasmic components limiting for nuclear growth, we biochemically fractionated *X. laevis* egg extract and identified the

histone chaperone nucleoplasmin (Npm2) as a putative nuclear size-scaling factor. Nuclear size increased in extract droplets supplemented with Npm2 protein, and embryos microinjected with Npm2 exhibited larger nuclei at gastrulation. Consistent with Npm2 being a nuclear-size scaling factor, per cell amounts of nuclear Npm2 and histone H2B decrease over development. Chromatin staining and micrococcal nuclease digestion assays showed that Npm2 increases chromatin compaction and nucleosome packing while decreasing the amount of euchromatin. We propose that Npm2 drives nuclear growth by altering chromatin organization. To further investigate how chromatin organization might influence nuclear size, we studied nuclei assembled de novo in *X. laevis* egg extract. Nuclei treated with DNase or MNase were still import-competent but failed to expand and properly localize lamins to the inner nuclear membrane, suggesting that nuclear import is not sufficient for nuclear growth. We are currently testing the model that chromatin stiffness produces intranuclear forces that contribute to nuclear growth. In summary, we propose that reductions in cell volume with concomitant decreases in the amounts of Npm2 and nuclear histones contribute to developmental nuclear size-scaling by affecting chromatin organization.

P389/B398

RanGAP Targeting to the Nuclear Envelope Is Essential for Development in *Drosophila*.

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The Ran GTPase is critical for nuclear-cytoplasmic transport, nuclear envelope (NE) assembly, and mitotic spindle formation. These processes are driven by gradients of GTP-bound Ran (Ran-GTP) and GDP-bound Ran (Ran-GDP). During interphase, Ran's chromatin-bound nucleotide exchange factor, RCC1, maintains high levels of Ran-GTP within nuclei. Conversely, Ran's GTPase activating protein, RanGAP, resides in the cytoplasm and promotes Ran-GTP hydrolysis, so that Ran-GDP is predominant in the cytosol. In fungi, RanGAP is diffusely distributed throughout the cytosol. However, multicellular organisms localize RanGAP to the NE. In vertebrates, RanGAP becomes SUMOylated, facilitating its association to the nucleoporin RanBP2 on the cytoplasmic face of the nuclear pore complex (NPC).We used cultured mammalian cells and *Drosophila to* investigate the functional consequences of RanGAP localization in metazoans. We used CRISPR/Cas9 gene editing to mutate human RanGAP (hRanGAP) in DLD-1 cells, preventing its SUMOylation and NPC targeting. Surprisingly, this change had no impact on cell viability and caused no obvious perturbations of nuclear transport or mitosis. The mechanism of *Drosophila* RanGAP (dRanGAP) targeting at the nuclear envelope had not been reported. We found that while dRanGAP associates to the NPC through binding to dRanBP2, the targeting mechanism is distinct from mammals to the extent that it occured via a direct association between dRanGAP and dRanBP2 without involvement of SUMOylation. We identified the domains in both proteins that mediate their binding and used CRISPR/Cas9 gene editing to generate dRanBP2 mutants (dRanBP2^{short}) that abolish dRanGAP anchorage to NPCs through a 23-amino-acid deletion. Homozygous RanBP2^{short} mutants exhibited no apparent growth defects as larvae. However, they died as pupae without gas bubble expulsion or eversion of the cephalic complex. This developmental arrest was rescued by a direct fusion of dRanGAP to dRanBP2^{short}, indicating that recruitment of dRanGAP to dRanBP2 per se was necessary for the pupal ecdysis sequence during development.Collectively, our results indicate that while the localization of RanGAP to the NE is widely conserved in multicellular organisms, the targeting mechanisms are not. Further, we find a previously unreported requirement for this localization in critical

tissue developmental processes, and we are currently working to understand the precise molecular role of dRanBP2-dRanGAP interaction during metamorphosis.

Nuclear Lamina and Laminopathies

P390/B399

Erk1/2 Phosphorylation of FHOD Connects Signaling and Nuclear Positioning Alternations in Cardiomyopathy Caused by Lamin A/C Gene Mutations.

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Lamin a and C, encoded by *LMNA*, are components of the nuclear lamina and contribute to the physical and biochemical properties of the nucleus. Mutations in *LMNA* cause striated muscle diseases including muscular dystrophy and dilated cardiomyopathy with a high risk of cardiac sudden death. Expression of muscle disease-causing lamin a variants leads to upregulation of ERK1/2 activity in hearts of humans and mice and inhibition of ERK1/2 ameliorates disease symptoms in mice. In a fibroblast model system, expression of muscle disease-causing lamin a variants disrupts nuclear positioning. We now show that striated muscle cells from *Lmna*$^{H222P/H222P}$ mice, which mimic human myopathies caused by *LMNA* mutation, have mis-positioned nuclei. In binucleated cardiomyocytes, the two nuclei were closer in *Lmna*$^{H222P/H222P}$ mice than in wild type mice and were not equidistantly spaced. In multinucleated skeletal muscle fibers, peripheral nuclei were clustered in *Lmna*$^{H222P/H222P}$ mice in contrast to the equidistant distribution in wild type mice. Using a fibroblast model, we found that muscle disease-causing lamin a variants inhibit actin-dependent nuclear movement and increase ERK1/2 phosphorylation of FHOD1/3, members of the formin family of actin regulators that bind nesprin-2 and at least for FHOD1 play a role in nuclear movement. Phosphomimetic forms of FHOD1/3 failed to rescue defective nuclear movement in FHOD1-depleted fibroblasts, whereas unphosphorylatable forms rescued the nuclear movement defect in fibroblasts expressing a muscle disease-causing lamin a variant. ERK1/2 phosphorylation of FHOD1/3 inhibited their F-actin bundling activity without affecting their actin polymerization activity. Phosphorylation of FHOD3, the major isoform in cardiomyocytes, was increased in hearts from *Lmna*$^{H222P/H222P}$ mice and reduced in mice treated with drugs that inhibit ERK1/2. Our results identify FHOD1/3 as the link between upregulated ERK1/2 activity and nuclear mis-positioning in muscle disease caused by *LMNA* mutations. They further suggest that nuclear mis-positioning may contribute to cardiac and skeletal muscle pathology.

P391/B400

Different Impact of Emerin in Skeletal and Cardiac Muscles from *Lmna*$^{H222P/H222P}$ Double Mutant Mice.

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Laminopathies are tissue-selective diseases that affect differently in organ systems. Mutations in nuclear envelope proteins, emerin (*EMD*) and lamin A/C (*LMNA*) genes, cause clinically indistinguishable myopathy called Emery-Dreifuss muscular dystrophy (EDMD) and limb-girdle muscular dystrophy. Several murine models for EDMD have been generated; however, emerin-null (Emd) mice do not show obvious skeletal and cardiac muscle phenotypes, and Lmna$^{H222P/H222P}$ mutant (H222P) mice show only a mild phenotype in skeletal muscle when they already have severe cardiomyopathy. Thus, the underlying molecular mechanism of myopathy due to nuclear envelopathy is still unclarified. We generated double

mutant (Emd-/-/Lmna$^{H222P/H222P}$; EH) mice to characterize muscle involvement and to elucidate roles of interactions between emerin and lamin A/C in skeletal and cardiac muscles. As H222P mice, EH mice grow normally and have breeding productivity. EH mice did not have morphological abnormalities in different organ systems, such as kidney, lung and reproductive organs; however, they showed gradual body weight loss after 18 weeks of age associated with progression of cardiomyopahty. EH mice showed severer muscle involvement compared with that of H222P mice which was an independent of cardiac involvement at 12 weeks of age. Nuclear abnormalities, reduced muscle fiber size and increased fibrosis were prominent in skeletal muscle from EH mice. Muscle phenotypes were aggravated in an age-dependent manner. Both H222P and EH mice had similar cardiac abnormalities at 30 weeks of age which indicates that emerin has been implicated to contribute to skeletal or cardiac muscle in a manner different to that of lamin A/C. Roles of emerin and lamin A/C in satellite cell function and regeneration of muscle fibers were also evaluated by cardiotoxin-induced muscle injury. Delayed increases in *Myog* and *Myh3* expression were seen in both H222P and EH mice; however, at day 7 after injury, the expression levels of those genes were similar with control and regenerated muscle fiber size was not different. EH mice showed the progression of muscular dystrophy before appearance of cardiac dysfunction similar to EDMD patients. These results indicate that EH mouse is a suitable model for studying skeletal muscle involvement, independent of cardiac function, and roles of interaction between emerin and lamin A/C in different tissues.

P392/B401

An Induced Pluripotent Stem Cell Model of *LMNA*-associated Dilated Cardiomyopathy to Study the Disease Pathogenesis.

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The *LMNA* gene encodes the intermediate filament proteins Lamins a and C, which form the nuclear lamina: a meshwork inside of the inner nuclear membrane that gives the nucleus structural support and modulates the organization of chromatin and other nuclear proteins. *LMNA* mutations cause numerous diseases, termed laminopathies, including dilated cardiomyopathy (*LMNA*-DCM) and muscular dystrophies. *LMNA*-DCM involves dilation of the left ventricle and thinning of the ventricle walls, which weakens the heart. On the cellular level, *LMNA*-DCM and skeletal muscle laminopathies are characterized by structural defects resulting from mechanically weakened nuclei that cannot adequately respond to mechanical forces, and by disrupted signaling pathways; however, the precise molecular mechanisms underlying the disease pathology remain unknown. In vitro models of *LMNA*-DCM are lacking, because primary cardiomyocytes are challenging to isolate and short-lived in culture. Thus, we aimed to develop an induced pluripotent stem cell-derived cardiomyocyte (iPSC-CM) model with *LMNA*-DCM patient-derived cells to study the cellular pathology. We found that *LMNA*-DCM iPSC-CMs have increased nuclear deformities compared to healthy controls, including abnormal nuclear shape and nuclear envelope blebbing, recapitulating defects found in cardiac tissue of laminopathic models. Furthermore, using a nuclear rupture reporter system, in which a GFP is fused to a nuclear localization signal (NLS-GFP), we observed nuclear rupture in *LMNA*-DCM iPSC-CMs, indicated by NLS-GFP spilling into the cytoplasm. Nuclear rupture exposes nuclear material, including chromatin, to the cytoplasm and can cause DNA damage. Accordingly, we found that *LMNA*-DCM iPSC-CMs had increased levels of

γH2AX, a marker of double stranded DNA breaks. In conclusion, our work demonstrates that patient-derived iPSC-CMs serve as a valuable in vitro model of *LMNA*-DCM. Ongoing work is aimed at investigating the functional effects of disrupted nucleo-cytoskeletal force transmission and structural damage to nuclei, as well as altered signaling pathways and DNA damage, in hopes of finding potential treatment avenues for *LMNA*-DCM.

P393/B402

The Use of Emerin-null and EDMD-causing Emerin Mutant Myogenic Progenitors to Elucidate the EDMD Mechanism.

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Mutations in the gene encoding emerin (EMD) cause Emery-Dreifuss muscular dystrophy (EDMD1), an inherited disorder characterized by progressive skeletal muscle wasting, irregular heart rhythms and contractures of major tendons. Emerin is a ubiquitously expressed integral inner nuclear membrane protein. The skeletal muscle defects seen in EDMD are caused by failure of muscle stem cells to differentiate and regenerate the damaged muscle. However, the underlying mechanisms remain poorly understood. RNA-sequencing performed on differentiating wildtype (WT) and emerin-null ($EMD^{-/y}$) myogenic progenitors revealed 1,945 genes were differentially expressed during the transition from differentiation day 0 to day 1 (transcriptional reprograming for myogenic commitment) between WT cells and $EMD^{-/y}$ cells. Pathways enriched in differentially expressed genes were identified using multiple bioinformatics platforms. TGF-β, IGF-1, p38 and ERK MAPK, JAK-STAT, VEGF, FGF, YAP/TAZ, cell cycle, integrin signaling, stem cell differentiation, muscle development and growth hormone signaling pathways were highly enriched in differentially expressed genes. We created stable myogenic progenitors expressing WT emerin and each EDMD-causing missense emerin mutant in an $EMD^{-/y}$ background to test if these mutant progenitors exhibited impaired myogenic differentiation. We also posited similar transcriptional reprograming defects would be seen in the EDMD emerin mutants. Importantly, WT emerin expression rescued $EMD^{-/y}$ differentiation. All EDMD mutant myogenic progenitors failed to rescue $EMD^{-/y}$ myogenic differentiation. RNA-sequencing using the DNBseq platform was done during differentiation of $EMD^{-/y}$ progenitors expressing WT emerin and each EDMD-causing emerin mutant (S54F, Q133H and Δ9599) and a control emerin mutation (M179) to identify changes in the transcriptional program contributing to their impaired differentiation. We anticipate these studies will further refine the molecular pathways implicated in EDMD1, which will be confirmed using small molecule inhibitors or activators of these pathways.

P394/B403

Defining the Sun1 Protein Interactome in Muscle Differentiation.

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The nuclear lamina is composed of A- and B-type lamins, which are intermediate filaments that assemble into a complex, insoluble meshwork beneath the inner nuclear membrane (INM). This, together with other nuclear envelope (NE) proteins, have diverse functions in genome tethering, transcriptional regulation and nuclear positioning. The latter can be attributed to a NE-spanning group of proteins known as the LINC complex. INM SUN-domain proteins interact with their outer nuclear

membrane counterparts, KASH-domain proteins, in the perinuclear space. Together, the LINC assembly provides an uninterrupted mechanical connection anchoring the nuclear lamina to the cytoskeleton. Sun1 is crucial in several developmental processes as well as being implicated in muscle-wasting disorders both as a causative and disease modifying component. *Lmna*, encoding lamins a and C, is widely expressed in somatic cells, yet mutations give rise to a range of tissue-specific diseases, including muscular dystrophies, for reasons not fully understood. Interestingly, loss of Sun1 in *Lmna* mutant mouse lines suppresses many of the *Lmna*-associated disease phenotypes. One possible explanation is that the nucleoplasmic protein interactions of Sun1 change in a tissue-specific manner, in response to the *Lmna* mutation present, to elicit disease phenotypes. To elucidate the Sun1 protein interactome, we used 2C-BioID, a proximity biotinylation proteomics procedure. It improves on the original BioID method by using a two component (FKBP-FRB) system to both enhance the correct localisation of the fusion protein and reduce false positives acquired through non-specific biotinylation. Here, details of the validation of the 2C-BioID system, as well as an *ex vivo* study using a FRB-Sun1 bait during myoblast differentiation are presented.

P395/B404

Disrupting the Nesprin-1 KASH Domain Suppresses Pathology of Lamin A/C Loss in the Heart.

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Mutations in the nuclear envelope (NE) proteins lamin A/C, encoded by *LMNA*, result in a variety of diseases, of which the most predominant is dilated cardiomyopathy (DCM). Among other NE proteins are the SUN and KASH domain proteins that form the LINC (Linker of Nucleoskeleton and Cytoskeleton) complexes. In the LINC complex, SUN-KASH domain interactions anchor 1 of 6 KASH domain proteins, including Nesprin-1, to the NE. In addition to *LMNA*, *SYNE1*, that encodes Nesprin-1, has been reported to be mutated in DCM. To investigate Nesprin-1 function, we used CRISPR/Cas9 to direct deletions to the 3' end of the murine *Syne1* gene, generating a frameshift mutation in the last coding exon of the KASH domain. As expected, in cells derived from homozygous *Syne1*^{Kfs} (KASH frameshift) mice, Nesprin-1 was dispersed from the NE. Consistent with another previously described *Syne1* mutant mouse line, but differing from 2 other independently derived lines, no obvious phenotypic defects were observed in our *Syne1*^{Kfs} homozygotes. To elucidate potential interactions between *Syne1* and *Lmna* in DCM, we crossed our *Syne1*^{Kfs} mice with *Lmna* mice where *Lmna* had either been globally deleted or where *Lmna* was inducibly ablated in cardiomyocytes. *Lmna* global knockout mice die within 3 weeks after birth but survive for up to 6 weeks on a homozygous *Syne1*^{Kfs} background. In contrast, loss of function of the closely related *Syne2* gene does not rescue global deletion of *Lmna*. In mice, in which tamoxifen-induced Cre/lox recombination resulted in deletion of *Lmna* in cardiomyocytes, death occurs within a month due to acute heart failure. On a homozygous *Syne1*^{Kfs} background, cardiomyocyte-specific *Lmna* null mice survive for at least 6 months following tamoxifen-induced deletion. This demonstrates that disrupting Nesprin-1-containing LINC complexes in cardiomyocytes ameliorates the pathological effects of the *Lmna* deletion. We propose that cytoskeletal forces transmitted by the LINC complex impinge on stress-sensitized *Lmna* mutant nuclei to cause DCM, and dispersal of these forces by mislocalization of Nesprin-1 relieves *Lmna* null nuclei from cytotoxic mechanical stress. We are investigating which cytoskeletal elements may contribute to this force transmission by immunofluorescence analysis to examine the NE localization of candidate proteins in *Syne1*^{Kfs} cardiomyocytes.

P396/B405

Defining Substrate Requirements for Cleavage of Farnesylated Prelamin a by the Integral Membrane Zinc Metalloprotease ZMPSTE24.

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The zinc metalloprotease ZMPSTE24 is critical for human health. ZMPSTE24 mediates proteolytic maturation of prelamin A, the farnesylated precursor of the nuclear scaffold protein lamin A. Uncleaved, permanently farnesylated prelamin a accumulates when ZMPSTE24-dependent processing of prelamin a is disrupted by mutations in the genes encoding either the prelamin a substrate or the protease (*LMNA* or *ZMPSTE24,* respectively). This accumulation causes the premature aging disease Hutchinson Gilford Progeria Syndrome (HGPS) and other related progeroid disorders. Defective prelamin a cleavage may also play a role in physiological aging, since ZMPSTE24 activity may diminish as we age. ZMPSTE24 is an integral membrane protease with a novel structure, distinct from other membrane proteases. Its seven membrane spans form a hollow chamber enclosing an interior catalytic site with narrow side portals through which the substrate accesses the chamber. Interestingly, prelamin a is the only known mammalian substrate for ZMPSTE24; however, the basis of this specificity remains unclear. To define the requirements for recognition of farnesylated prelamin by ZMPSTE24, we used a humanized yeast system. We performed a comprehensive mutagenesis scan of the eight residues in prelamin a flanking the ZMPSTE24 cleavage site (TRSY/LLGN), mutating them to encode all other 19 possible residues. These studies allow us to generate a heatmap of cleavage efficiency that indicates the importance of residues in the P1' and P2' positions, just C-terminal to the cleavage site and begins to define a consensus motif with potential predictive value to identify other ZMPSTE24 substrates. In yeast, as in mammalian cells, the C-terminal 41 amino acids of prelamin a contain sufficient information for ZMPSTE24 cleavage. We have analyzed the 23-residue long region N-proximal to the cleavage site, as well as the 14 residues between the cleavage site and the farnesylated Cys, by generating a series of alanine substitutions, alanine additions, and deletions in prelamin A. This work indicates a surprising flexibility in the features required for recognition of prelamin a by ZMPSTE24. This flexibility must be reconciled with ZMPSTE24's selectivity for prelamin A. We expect that elucidating the substrate requirements of ZMPSTE24 could provide insight into integral membrane protease function and have valuable therapeutic implications for both progeroid disorders and healthy aging.

P397/B406

Role of E3 Ubiquitin Ligase RNF123 in Pathophysiology of Laminopathies.

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Lamin A/C is a type V intermediate filament protein present underneath the inner nuclear membrane in metazoan cells and plays an important role in maintaining the structural integrity of the nuclear lamina. A-type lamins associate with the other nuclear proteins, such as lamin B1, emerin, retinoblastoma (pRb), PCNA, and lamina-associated polypeptide (LAP) 2α to perform essential nuclear functions, such as chromatin organization and tethering of heterochromatin to the nuclear periphery, cell cycle progression and differentiation, and effective DNA damage repair. Hence, mutations in metazoan nuclear lamina proteins, mainly lamin A/C cause rare tissue-specific genetic disorders, collectively termed as laminopathies. The hallmarks of laminopathic cells include increased nuclear fragility, DNA

damage, and defective cell differentiation. Here, we identified the upregulation of a RING domain-containing E3 ubiquitin ligase RNF123 in cells expressing laminopathic mutations. We also observed that RNF123 interacts with lamin a and lamin B1, and lamin-binding proteins, such as pRb, LAP2α, and emerin. Overexpression of RNF123 increased the proteasomal turnover of these nuclear proteins, an effect that was reversed upon the downregulation of this ligase. Increased proteasomal degradation of these nuclear proteins disturbs the homeostatic nuclear functions and possibly alters nuclear structure and function, thereby predisposing cells to fragile nuclei, increased DNA damage, and altered cell cycle kinetics. Laminopathic cells also show similar phenotypes, suggesting that increased ubiquitination and proteasomal turnover of nuclear proteins due to upregulation of E3 ligase RNF123 contributes to cellular pathophysiology observed in laminopathic cells.

P398/B407

Power Law Relationships in Expression Profiles Link Lamin-B to Proliferation and Poor Survival and Link Fibrosis to Prolonged Survival.

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Within organelles, cells, or tissues including tumors, spatiotemporal relationships between component molecules generally reflect principles that range from stoichiometric interactions or co-regulated genes to fractal organization. Such physico-chemical concepts are applied here in scaling analyses of key structural factors in the extracellular matrix and nucleus of liver, lung, and breast tumors in the Cancer Genome Atlas (TCGA) and in single cell RNA-Seq data. The nuclear filament LMNB1 exhibits systematic power laws with proliferation genes that are always high in tumors and predict poor survival. The strongest scaling is consistent with a lamina surrounding a rapidly replicating, fractal genome, and in *vitro* studies likewise show LMNB1 scales with and modulates cell cycle. The main fibrosis collagens *COL1A1 and COL1A2* also scale with each other, stoichiometrically, and with genes in a common fibrotic program across cancers. For liver tumors, the fibrotic genes surprisingly predict prolonged survival when liver tumors are more fibrotic than adjacent tissue. Basement membrane factors *COL4A1 and COL4A2* exhibit surface-to-volume type scaling with *COL1A1*, but noisy data generally frustrates such weak scaling. Single cell RNA-Seq data nonetheless exhibits scaling that underscores a simple means of validating expression data based on physicochemical principles.

P399/B408

How to Compartmentalize: the Role of Lamin C in Re-establishing Genome Organization After Cell Division.

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The contents of the eukaryotic nucleus are highly organized for functional efficiency. This can be seen on a variety of levels within the nucleus from protein-DNA hubs that perform particular roles, such as DNA damage repair, to higher organization of the chromatin itself. Recent studies using the chromosome conformation capture technique HiC have identified two types of 3D organization: areas of local self-interaction called topologically associated domains (TADs) and long-range genome-wide self-interactions that divide the genome into a (active) and B (inactive) compartments. Recently, we have shown that the B compartment and Lamin Associated Domains (LADs) represent the same functional

regions of the genome, including a shared sub-domain structure and boundaries. LADs are largely inactive regions of chromatin that are proximal to the meshwork of lamins that underlie the nuclear membrane and are marked by specific repressive histone modifications. Recently, we and others have demonstrated that, at the single cell level, LADs, along with TADs and A/B compartments are lost during mitosis and the chromosomes are reorganized and undergo large scale compaction. All genome organization is reestablished after mitosis during G1, but a great deal is still unknown about how this dynamic reorganization happens. Using the m6Atracer system, which allows us to image LADs in live cells, we have observed that LAD self-association occurs early in G1 prior to LAD establishment at the lamina. All of the LADs within a chromosome form 1-2 aggregates during early G1 and the nonLADs/A compartment of the chromosome form an exterior layer around these aggregates. These aggregates then make their way to the nuclear lamin over the course of several hours where they become anchored. How these aggregates are formed remains largely unknown but we have evidence showing that lamin C may be play a critical role in anchoring the LAD aggregates at the nuclear periphery.

P400/B409

Protein Kinase C Activity Alters Lamin B1 Dynamics Differently in Induced Pluripotent Stem Cells and Germ Layer Cells.

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Cellular differentiation is fundamental to the growth and development of multicellular species. There are many changes in the composition of organelles and cellular structures during differentiation that are not well understood. We have been focusing on the nuclear lamina. While expression levels of lamins and lamina-associated proteins are known to vary in different tissues, the exact nature and possible significance of these changes during the differentiation process are not yet fully understood. In this study, we analyze some of the basic changes that occur in the nuclear lamina as human induced pluripotent stem cells (iPSCs) differentiate into cells of the three main germ cell layers. We have previously observed that the lamin B1 dynamics in each of these cell types vary significantly. In particular, iPSCs and mesoderm laminas have significantly higher lamin B1 dynamics than laminas of the endoderm or ectoderm, suggesting that there are innate structural differences in the laminas of these cells. To begin to address the mechanistic basis for these differences, we measured lamin B1 dynamics in response to altered PKC activity. We observed by lamin B1 FRAP analysis that PKC activation decreases the rate of fluorescence recovery in iPSCs by ~60% while PKC inhibition increases the rate of fluorescence recovery over 2.5-fold. Furthermore, PKC activation increases the total fluorescence recovery in mesoderm cells by ~10% while inhibition decreases total fluorescence recovery by ~30%. These data suggest that PKC-dependent nuclear lamin dynamics are altered in early differentiation. To address how these changes in lamin dynamics might affect the composition of the nuclear lamina, we are currently characterizing the lamin B1 interactome in stem and differentiated cells by co-immunoprecipitation and mass spectrometry.

P401/B410

Simplified Method for the Identification of Proteins, Rna and Dna at the Nuclear Envelope.

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The nuclear lamina (NL) is a stable proteinaceous interface between the genome and the rest of the cell. This structure has garnered considerable interest due to the finding of diseases caused by NL

components and the lethal phenotype of most null animal models. Historically, the study of NL has proved difficult, partly due to the biochemical nature of the NL. This has led to a proliferation of surrogate methods that employ the proximity tagging strategy. Here, we describe a fast, robust and simplified method to identify proteins, RNA, and DNA associated with the NL. This method uses the Ascorbate Peroxidase (APEX) enzyme linked to Lamin B1. The discrimination between protein, RNA and DNA is based entirely on the application of RNase, DNase or protease after purification. We demonstrate that the method identifies many proteins previously known to be at the NL, in addition to new protein components. We show that RNA species associated with the NL are largely spliced and that association with the NL is related primarily to expression level. Finally, we show that lamina-associated domains (LADs) can be readily mapped and do not change with cell cycle stage. This simplified method should be a valuable tool for the study of the basic and medical aspects of the NL.

P402/B411

Emerin and Nuclear Structure of Invasive Breast Cancer Cells.

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Dysmorphic nuclei are commonly used for diagnosis and prognosis of many different types of cancer. However, the underlying mechanism regulating these structural changes is poorly understood. It is becoming clear nuclear envelope proteins play an important role in regulating nuclear size and structure in cancer. Altered expression of nuclear lamins were found in many cancers and higher mean levels of lamins were associated with better clinical outcomes. The nucleus is the largest organelle in the cell with a diameter between 3-10 um. Nuclear size can greatly impact the cell's ability to migrate due to its large size and rigidity. Recent studies showed nuclear size, stiffness, and deformability is regulated by expression of nuclear envelope proteins, including emerin and other nuclear lamina proteins. Here we began to test emerin's role in nuclear size and stiffness and its effect one the invasiveness of breast cancer cells. Here we show the invasive breast cancer cell lines, MDA-231 and MDA-157 had a 1.4 to 1.7-fold smaller nuclei than normal breast tissue cells. These breast cancer cell lines had 1.4 to 1.8-fold decrease in emerin expression when compared to normal breast cells. Overexpression of emerin in invasive cancer cells increases nuclear size by 1.4-fold. Emerin expression also impairs their migration through 8 um pores. Emerin mutants that specifically inhibit binding to selected partners were tested for rescue in nuclear size and migration. Emerin mutants specifically disrupting binding to HDAC3, actin, or lamin a failed to rescue nuclear size. Emerin mutants specially disrupting binding to transcription regulators or BAF rescued nuclear size and migration. Thus, we concluded that interaction of emerin with the nucleoskeleton plays a significant role in regulating nuclear structure during cancer cell transformation, with chromatin interactions via HDAC3, surprisingly, also contributing to nuclear structure. These results align with previous studies showing both the lamins and chromatin contribute to nuclear structure. iRFP and GFP-EMD co-expressing cell lines were made to monitor metastases formation in mice. Each emerin mutant was co-expressed with iRFP to test if the functional interactions with the nucleoskeleton or chromatin (via HDAC3) is important for metastasis.

P403/B412

Histone Acetyltransferase Inhibition Rescues Emerin-null Myogenic Differentiation.

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Emery-Dreifuss Muscular Dystrophy (EDMD) is a disease characterized by skeletal muscle wasting, contractures of major tendons, and cardiac conduction defects. Compromised skeletal muscle regeneration is predicted to result from impaired muscle stem cell differentiation. Mutations in the gene encoding emerin cause EDMD1. We previously showed emerin-null myogenic progenitors fail to properly exit the cell cycle, exhibit decreased myosin heavy chain expression, and have decreased myotube formation. Treatment with theophylline, a HDAC3 activator, rescued myotube formation in differentiating emerin-null myogenic progenitors. This suggested emerin activation of HDAC3 activity to reduce H4K5 acetylation is important for myogenic differentiation. Pharmacological inhibitors of histone acetyltransferases targeting acetylated H4K5 (Nu9056, L002) were used to test if the increased acetylated H4K5 was responsible for inhibiting emerin-null myogenic differentiation. Nu9056 and L002 rescued emerin-null myogenic progenitor differentiation. Treatment with SRT1720, which targets the NAD^+-dependent deacetylase SIRT1, showed no significant change in myotube formation. Thus, we conclude emerin regulation of HDAC3 activity to affect H4K5 acetylation dynamics is important for myogenic differentiation. Targeting H4K5ac dynamics represents a new strategy for ameliorating the skeletal muscle wasting seen in EDMD. The widely used COPD drug theophylline, which activates HDAC3 and rescues emerin-null differentiation, is a prime candidate for future testing.

P404/B413

Rescue of DNA Damage After Constricted Migration Reveals a Mechano-regulated Threshold for Cell Cycle.

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Migration through 3D constrictions can cause nuclear rupture and mis-localization of nuclear proteins, but damage to DNA remains uncertain as does any effect on cell cycle. Here, myosin-II inhibition rescues rupture and partially rescues the DNA damage marker γH2AX, but an apparent block in cell cycle appears unaffected. Co-overexpression of multiple DNA repair factors or antioxidant inhibition of break formation also exert partial effects, independent of rupture. Combined treatments completely rescue cell cycle suppression by DNA damage, revealing a sigmoidal dependence of cell cycle on excess DNA damage. Migration through custom-etched pores yields the same damage threshold, with ~4μm pores causing intermediate levels of both damage and cell cycle suppression. High curvature that is rapidly imposed by pores, probes, or small micronuclei consistently associates nuclear rupture with near-instantaneous dilution of lamin-B, whereas dilution of lamin-A is time-dependent, consistent with lamin-A's proposed viscous properties. Lamina dilution associates with nuclear rupture based on entry from cytoplasm of chromatin-binding cGAS (cyclic-GMP-AMP-synthase) as well as loss of repair factors. The cell cycle block caused by constricted migration is nonetheless reversible, with a potential for DNA mis-repair and genome variation.

P405/B414

Mutant Lamins Cause Nuclear Envelope Rupture and DNA Damage in Skeletal Muscle Cells.

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Mutations in the human *LMNA* gene, which encodes the nuclear envelope proteins lamins a and C, cause autosomal dominant Emery-Dreifuss muscular dystrophy, congenital muscular dystrophy, limb-girdle muscular dystrophy, and other diseases collectively known as laminopathies. The molecular mechanisms responsible for these diseases remain incompletely understood, but the muscle-specific defects suggest that mutations may render nuclei more susceptible to mechanical stress. Using three mouse models of muscle laminopathies, we found that *Lmna* mutations caused extensive nuclear envelope damage, consisting of chromatin protrusions and transient rupture of the nuclear envelope, in skeletal muscle cells *in vitro* and in *vivo*. The nuclear envelope damage was associated with progressive DNA damage, activation of DNA damage response pathways, and reduced viability. Intriguingly, nuclear envelope damage resulted from nuclear movement in maturing skeletal muscle cells, rather than actomyosin contractility, and the nuclear damage was reversed by either depletion of kinesin-1 or disruption of the Linker of Nucleoskeleton and Cytoskeleton (LINC) complex. Depletion of kinesin-1 or LINC complex disruption reduced DNA damage, indicating that DNA damage is the result of nuclear envelope damage. Furthermore, LINC complex disruption rescued myofiber function and viability, indicating that the myofiber dysfunction is the result of mechanically induced nuclear envelope damage. The extent of nuclear envelope damage and DNA damage in the different *Lmna* mouse models strongly correlated with the disease onset and severity in *vivo*, and inducing DNA damage in wild-type muscle cells was sufficient to phenocopy the reduced cell viability of lamin A/C-deficient muscle cells, suggesting a causative role of DNA damage in disease pathogenesis. Corroborating the mouse model data, muscle biopsies from patients with *LMNA* associated muscular dystrophy similarly revealed significant DNA damage compared to age-matched controls, particularly in severe cases of the disease. Taken together, these findings point to a new and important role of nuclear envelope rupture and DNA damage as pathogenic contributors for these skeletal muscle diseases.

P406/B415

Rescue of Migration-induced Nuclear Rupture by Myosin-II Inhibition Depends on Lamin-A.

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During tumor growth and metastasis, cancer cells squeeze through micron-sized pores, invade basement membrane barriers, and enter blood capillaries. Such migration through rigid micropores has been shown to cause nuclear lamina and envelope rupture, which may result in increased DNA damage due to mis-localization of DNA repair factors. Reduction of actomyosin force can rescue migration-induced nuclear rupture, but it remains unclear whether this rescue depends on levels of lamin-A, a main nucleoskeletal protein which stiffens and strengthens the nucleus but is down-regulated in multiple cancers, including lung and breast cancers. Given that lamin-A suppression down-regulates actomyosin contractility but can also increase migration rate of the softer nuclei, we investigated whether lamin-A levels modulate the potential effects of actomyosin inhibition on nuclear rupture in constricted migration. Here, wild-type (WT) and lamin-A knockdown (KD) U2OS human osteosarcoma cells were migrated through 3 μm or 8 μm pores. Compared to WT, lamin-A KD cells exhibited much less nuclear

envelope rupture—as indicated by nuclear bleb formation—after 3 μm pore migration. Meanwhile, myosin-II inhibition *via* blebbistatin reduced migration-induced nuclear rupture among both WT and lamin-A KD cells; however, the fold-decrease in nuclear rupture was lower for KD versus WT. Thus, when lamin-A is knocked down, myosin-II inhibition only partially rescues migration-induced rupture, suggesting that lamin-A might play a role in the rescue mechanism. The results imply that tumors with low lamin-A (e.g. lung and breast) will not show more nuclear rupture and more damage in *in vivo* migration, and that recent reports of higher DNA damage in model tumors with low lamin-A could reflect nuclear rupture because of a stiff microenvironment.

P407/B416

Dyt1 Dystonia Patient-derived Fibroblasts Have Increased Deformability and Susceptibility to Damage by Mechanical Forces.

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DYT1 dystonia is a neurological movement disorder that is caused by a loss-of-function mutation in the *DYT1*/*TOR1A*gene, which encodes torsinA, a conserved luminal ATPases-associated with various cellular activities (AAA+) protein. TorsinA is required for the assembly of functional linker of nucleoskeleton and cytoskeleton (LINC) complexes, and consequently the mechanical integration of the nucleus and the cytoskeleton. Despite the potential implications of altered mechanobiology in dystonia pathogenesis, the role of torsinA in regulating cellular mechanical phenotype, or mechanotype, in DYT1 dystonia remains unknown. Here, we define the deformability of mouse fibroblasts lacking functional torsinA as well as human fibroblasts isolated from DYT1 dystonia patients. We find that the deletion of torsinA or the expression of torsinA containing the DYT1 dystonia-causing ΔE302/303 (ΔE) mutation results in more deformable cells. We observe a similar increased deformability of mouse fibroblasts that lack lamina-associated polypeptide 1 (LAP1), which interacts with and stimulates the ATPase activity of torsinA in *vitro*, as well as with the absence of the LINC complex proteins, Sad1/UNC-84 1 (SUN1) and SUN2, lamin A/C, or lamin B1. Consistent with these findings, we also determine that DYT1 dystonia patient-derived fibroblasts are more compliant than fibroblasts isolated from unafflicted individuals. DYT1 dystonia patient-derived fibroblasts also exhibit increased nuclear strain and decreased viability following mechanical stretch. Taken together, our results establish the foundation for future mechanistic studies of the role of cellular mechanotype and LINC-dependent nuclear-cytoskeletal coupling in regulating cell survival following exposure to mechanical stresses.

P408/B417

The Exocyst Complex Is Required to Maintain Mammalian Nuclear Architecture.

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The highly-conserved exocyst complex plays key roles in vesicle transport and establishment of polarity. It predominantly localises to sites of active cell growth. However, there is emerging evidence that the exocyst functions in other cellular contexts. Here, we show that the exocyst is also present in the nucleus, where it influences multiple aspects of nuclear architecture. The exocyst exhibits both perinuclear and nucleoplasmic localisations in mammalian cells by immunofluorescence. Further investigation by immuno-electron microscopy reveals that exocyst members localise to both the inner

and outer faces of the nuclear envelope, as well as to electron-dense regions within the nucleoplasm. GFP-TRAP analysis of multiple exocyst subunits identifies potential interactions with proteins of the inner nuclear envelope and nuclear lamina, further reinforcing the idea of a nuclear function of the exocyst. To investigate the nuclear effects of reduction of the exocyst, we used both CRISPR-mediated gene editing and siRNA against three individual exocyst subunits. Reduction in exocyst protein expression results in abnormal nuclear morphology, and disrupted organisation of the inner nuclear envelope and lamina. We demonstrate by immunofluorescence and electron microscopy that heterochromatin distribution is also altered. We conclude that the exocyst is required for correct nuclear architecture, and that this role likely has wider implications for gene expression and DNA repair.

Endocytosis 1

P409/B419

Mechanobiology of Endocytic Vesicle Formation an alyzed by Sla2 Force Sensors.

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Mechanical forces exerted by multiprotein machines are indispensable for many vital cellular processes. One of the best-studied examples is membrane reshaping during clathrin-mediated endocytosis, a principal vesicle trafficking route responsible for molecular uptake, signaling, and membrane homeostasis. During endocytosis, a small region of the plasma membrane reshapes from a flat sheet to a closed vesicle. This reshaping requires mechanical force, which is provided by multiple endocytic proteins and actin polymerization. Several theoretical models have been already proposed to describe force requirements during endocytosis (Lacy et al., 2018). However, to mechanistically understand force-dependent endocytic vesicle formation, applied forces need to be analyzed in vivo to report real force values and to delineate roles of involved proteins, and physical constraints.To achieve that, we used FRET (Förster Resonance Energy Transfer) tension sensors (Freikamp et al., 2016), which allow the measurement of pN forces in vivo, and inserted them into the yeast Hip1R protein Sla2. Sla2 is part of the essential Sla2-Ent1 (Hip1R- epsin 1-3 in human) protein linker transmitting force of polymerizing actin cytoskeleton to the plasma membrane (Skruzny et al., 2012, 2015). We followed forces transmitted over Sla2 in real time by measuring FRET changes of Sla2 force sensors during individual endocytic events. Using sensors of different force sensitivity, we estimated the maximal force applied on Sla2 to be higher than 9 pN. Next, we analyzed the role of individual endocytic factors and physical cues in force-dependent steps of endocytosis. We increased the size of the actin meshwork at the endocytic site by deleting the negative WASp regulator Bbc1, which caused the lessening of the force transmitted prior to vesicle scission. We also counteracted the high turgor pressure of yeast cytoplasm by increasing the medium osmolarity and observed an overall decrease in the force required for membrane invagination. Finally, current experiments monitor endocytic force requirements under conditions of altered plasma membrane tension and temperature. We believe that our data will form a base of comprehensive, experimental-based biomechanical model of endocytic vesicle formation, which could be also highly valuable for understanding of other force-dependent membrane reshaping processes in the cell.

P410/B420

Complimentary Action of ENTH and IDP Domains in Epsin Supports Clathrin-mediated Endocytosis in High Membrane Tension Condition.

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Cells live in and adapt to constantly changing physical environment and how changes in membrane tension regulate various cellular processes is a fundamentally important question. Epsin is an adaptor protein in clathrin-mediated endocytosis that aids in membrane bending. Two main domains in epsin, a N-terminal ENTH (epsin N-terminal homology) domain and a C-terminal IDP (intrinsically disordered protein) domain, have been shown to promote membrane bending via two distinct molecular mechanisms. ENTH domain insert its H0 alpha helix into the lipid bilayer to initiate membrane curvature while IDP domain causes membrane bending via steric repulsion. In this present work, we show that cells treated with a hypo-osmotic solution to increase membrane tension show an increase fraction of short-lived CCPs, suggesting a defect in clathrin assembly. However, overexpressing epsin rescues the stability of CCPs under hypo-osmotic shock. The recruitment of epsin increases in CCPs with elevated membrane tension by either increasing the area of cell spreading or by osmotic shock. Epsin gets recruited prior to clathrin to CCP nucleation sites and reaches the tension dependent maxima before clathrin assembly. However, masking the N-terminus of ENTH with EGFP or deletion of ENTH in epsin, delays recruitment of epsin to CCP nucleation site. Cells expressing ENTH-deleted epsin no longer have elevated recruitment of epsin at high tension. Moreover, the removal of H0 alpha helix in ENTH is responsible for the loss of tension sensitivity. ENTH domain, while necessary for tension sensitivity of epsin is not necessary for epsin puncta formation. However, deletion of AP2 binding and clathrin binding sites in IDP domain renders epsin cytosolic and without the ability to recruit to membrane. Further, IDP domain itself is sufficient for increasing stability of CCPs as expressing the IDP domain of epsin reduces the population of short-lived pits when recruited to CCPs. We conclude that H0 alpha helix in ENTH domain of epsin acts as a tension sensor and IDP domain acts as a tether stabilizing CCP complex. Our work reveals the complimentary action of ENTH and IDP domains of epsin allow it to support CME in high tension conditions.

P411/B421

ESCRT-dependent Protein Sorting Is Required for the Viability of Clathrin-mediated Endocytosis Mutants and the Maintenance of Plasma Membrane Protein Composition.

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Endocytic trafficking from the plasma membrane (PM) regulates many processes, including lipid and protein turnover, signaling, and nutrient uptake. During clathrin-mediated endocytosis (CME), adaptors bind to cytoplasmic regions of transmembrane cargo proteins, and many of these endocytic adaptors are also directly involved in the recruitment of clathrin. This clathrin-associated sorting protein family of adaptors includes the yeast epsins, Ent1/2, and AP180/PICALM homologues, Yap1801/2. Mutant strains lacking these four adaptors, but expressing a single epsin N-terminal homology (ENTH) domain necessary for viability (4Δ+ENTH), exhibit endocytic defects, such as cargo accumulation at the plasma membrane (PM). This CME-deficient strain provides a sensitized background ideal for revealing cellular components that interact with clathrin adaptors. We performed a mutagenic screen to identify alleles that are lethal in 4Δ+ENTH cells, a genetic interaction known as synthetic lethality, using a colony-

sectoring reporter assay. After isolating candidate synthetic lethal genes by complementation, we confirmed that mutations in *VPS4* lead to inviability of a 4Δ+ENTH strain. Vps4 mediates the final step of endosomal sorting complex required for transport (ESCRT)-dependent trafficking, and we found that multiple ESCRT components are also essential in 4Δ+ENTH cells, including Snf7, Snf8, and Vps36. Deletion of *VPS4* from an *end3*Δ strain, another CME mutant, similarly resulted in inviability, and upregulation of a clathrin-independent endocytosis pathway rescued 4Δ+ENTH *vps4*Δ cells. Loss of Vps4 from an otherwise wild-type background caused multiple cargoes to accumulate at the PM due to an increase in Rcy1-dependent recycling of internalized proteins to the cell surface. Additionally, *vps4*Δ *rcy1*Δ mutants exhibited deleterious growth phenotypes. Together, our findings reveal the previously unappreciated effects of disrupted ESCRT-dependent trafficking on endocytic recycling and the PM.

P412/B422

Unveiling the Dynamics of Intracellular Neonatal Fc Receptor-ligand Interactions in Primary Macrophages by Fluorescence Fluctuation Spectroscopy (FFS) and FLIM-FRET Microscopy.

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The neonatal Fc receptor (FcRn) is an MHC class I-like molecule expressed in several tissues, which interacts with the serum proteins IgG and albumin. FcRn binds endocytosed IgG and FcRn in a pH-dependent manner within intracellular endosomes. The ability to interact with FcRn under acidic conditions allows the sorting of IgG and albumin into endosomal recycling pathways and contributes to their high serum concentrations and uniquely long half-life. However, the molecular basis of the underlying trafficking events in the FcRn-dependent recycling system, especially for albumin, have yet to be resolved. Recent studies by our laboratory suggest that tubular transport carriers emerging from newly formed macropinosomes mediate a fast, FcRn-dependent recycling of albumin in bone marrow-derived macrophages (BMDMs). To characterize the intracellular interaction between albumin and FcRn in living cells and resolve the spatiotemporal dynamics of the albumin-FcRn recycling system in BMDMs, a multiplexed biophysical approach to fluorescent microscopy was established. Fluorescence fluctuation spectroscopy (FFS) of single fluorescent-labelled albumin molecules revealed a higher fraction of immobile albumin molecules within endosomal structures in the presence of FcRn compared to FcRn KO macrophages. To investigate the intracellular distribution of this interaction, the binding of a FcRn-mCherry fusion protein with A488-labelled albumin was spatially mapped using the phasor approach to FLIM-FRET. The presence of the FcRn fusion protein leads to a significantly reduced fluorescent lifetime of Alexa488-albumin molecules (2.5 ns) when compared to the absence of FcRn (3.3 ns). A FLIM-FRET time course following endocytosis of Alexa488-albumin demonstrated a gradual increase in the fraction of albumin molecules undergoing FRET from 20% to 70% during the first 15 minutes after endocytosis. Furthermore, this increase in FRET – which indicated high albumin-FcRn interaction – was observed within tubular transport carriers emanating from endosomal compartments. Collectively, these data unveil the spatiotemporal kinetics of FcRn-ligand binding and recycling. The approaches used give novel insights into the highly dynamic trafficking of albumin and FcRn within endosomal structures and also have wide applicability for the investigation of other intracellular ligand-receptor interactions.

P413/B423

Caveolae Stabilization by Ehd2 Is a Crucial Factor in Lipid Uptake and Obesity.

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Caveolae are membrane invaginations of 50 - 80 nm in size that are ubiquitously found in endothelial cells, adipocytes or fibroblasts. In addition to their function in transporting molecules across the cell membrane, caveolae are also involved in lipid metabolism, signal transduction and mechanoprotection. Besides caveolin and the cavin protein families, the dynamin-related ATPase Eps15 homology domain-containing protein 2 (EHD2) localizes to caveolae, particularly to their necks, therefore attaching them to the membrane. By combination of electron microscopy and tomography and TIRF imaging, we found that loss of EHD2 in vivo resulted in increased detachment and mobility of caveolae in adipocytes or fibroblasts. Furthermore, we found an increased fatty acid uptake and consequently enlarged lipid droplets in cells and tissue lacking EHD2. Detailed analysis of the fatty acid uptake mechanism revealed an involvement of the fatty acid transporter CD36 that partially localizes within the caveolar membrane structures. Obese patients and obesity mouse models further revealed downregulation of EHD2 and consequently increased detachment of caveolae from the plasma membrane. In summary, the observed results suggest that the EHD2-dependent stability of caveolae to the plasma membrane is a crucial factor in cellular lipid uptake.

P414/B424

A New Approach to Reconstruct Dynamics from Static Super-Resolution Images Reveals Structural Rearrangements of Proteins during Endocytosis.

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Clathrin-mediated endocytosis (CME) is a crucial cellular pathway that is involved in the uptake of molecules, membrane homeostasis and regulation of signalling. Budding yeast is a well-established model organism to study CME because of its easy genetics and the regular progression of CME. During CME in yeast, a protein machinery comprising more than 50 different proteins drives the invagination of the plasma membrane. One important part of the machinery is the actin network which provides the force for membrane invagination against the turgor pressure. Currently, the field is beginning to understand the architecture of the actin network and the function of its individual proteins involved in force generation. However, for example the nanoscale movement and orientation of the actin filaments is not fully understood and the mechanism by which actin crosslinkers function is postulated from modeling. To further elucidate the mechanisms of force generation we want to determine the spatial distribution of different endocytic proteins during membrane ingression with 10-20 nm resolution by dual-color high-throughput single-molecule localization microscopy (SMLM). The high resolution reached by SMLM requires acquisition times of several minutes which is why the cells have to be fixed prior to imaging. The fixation stops each endocytic site at a random time point along the endocytic timeline, hence dynamic information is lost. To overcome this problem, we developed an algorithm to reconstruct the endocytic time points of individual static snapshots by correlating them to temporally resolved data from living cells. For this we image the protein of interest in one color alongside with a

reference structure in a second color. We then use multi-dimensional features of this reference structure like height and intensity distribution to sort the individual snapshots temporally and align them spatially. We then calculate a running average of the sorted snapshots and combine different datasets to visualize the rearrangement of the different proteins. We have first results showing that our approach is able to spatially align the individual snapshots and sort them temporally based on geometric features of the reference structure. In a next step we are working on reproducing rearrangements of proteins of interest known from live-cell and electron microscopy. Finally, we will reconstruct the nanoscale rearrangement of key proteins of the endocytic machinery.

P415/B425

Metabolic Regulation of Adherens Junction Protein Trafficking in Rab11a Recycling Endosomes.

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Background: Vascular injury disrupts endothelial adherens junctions (AJs), which leads to increased vascular permeability, fluid accumulation in tissues and the influx of inflammatory cells. Following injury induced by inflammation or by other AJ-disrupting stressors, selected AJ proteins such as VE-cadherin and β-catenin are internalized and subsequently recycled back to the surface, thus restoring an intact barrier. Several mechanistic mediators of AJ recycling and trafficking have been identified but little is known about the metabolic regulation of this process. We recently identified the glycolysis regulatory enzyme PFKFB3 as an essential mediator of the endothelial response to injury. Here, we examine the role of PFKFB3 in regulating subcellular ATP production and its role in the trafficking of adherens junction proteins during acute injury as well as during its resolution. **Methods and Results:** to determine whether PFKFB3 regulated endothelial barrier restoration, we performed a transendothelial electrical resistance (TER) assay using Human Lung Microvascular Endothelial Cells (HLMVECs). PFKFB3 inhibition led to a 91% reduction in the rate of recovery following permeability induced by calcium chelation ($p<0.0001$), a non-inflammatory means of disrupting AJ integrity. Inhibition of PFKFB3 also markedly reduced surface levels of VE-cadherin following injury, indicating a key role of glycolysis in regulating the trafficking of VE-cadherin. Surprisingly, PFKFB3 inhibition did not affect the interaction between VE-cadherin and β-catenin as assessed by a proximity ligation assay, nor did it appear to affect the localization of these complexes at the cell junctions. We thus hypothesized that AJ proteins are trafficked to the plasma membrane in endocytic vesicles but are unable to fuse with the plasma membrane due to reduced regional ATP levels. In support of this hypothesis, we found that forced membrane targeting of PFKFB3 led to a 10% increase in ATP levels at the cell membrane ($p<0.005$), and subsequently a 22% increased rate of barrier restoration ($p<0.05$). Additionally, PFKFB3 inhibition led to accumulation of the recycling endosome marker Rab11a at cell junctions, which colocalized with VE-cadherin/β-catenin complexes. **Conclusions:** These findings show that spatial regulation of ATP production by PFKFB3 is necessary for the restoration of endothelial AJs following injury, indicating the importance of compartmentalized modulation of ATP levels and providing a better understanding of the metabolic regulation of AJ disassembly, trafficking and re-assembly. Modulating compartment-specific glycolysis could lead to the development of novel therapeutic approaches in which endothelial barrier function is acutely compromised.

P416/B426

Synuclein Regulates AP2-recruitment to the Membrane.

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α-Synuclein is a presynaptic protein whose mutation and aggregation is associated with several neurodegenerative disorders, including Parkinson's disease. It is highly expressed in the presynaptic compartment where it associates with synaptic vesicles. Recent studies indicate that α-synuclein regulates clathrin mediated synaptic vesicle endocytosis under physiological and pathological conditions. However, the molecular mechanism by which this occurs remains unknown. Here, we show that α-synuclein binds to isolated synaptic membranes in an ATP-dependent manner, similar to the clathrin adaptor protein AP2. In contrast, other endocytic proteins, such as clathrin heavy chain and the large GTPase dynamin-1 bind to synaptic membranes independently of ATP. This suggests that α-synuclein and AP2 share a common synaptic membrane recruitment pathway. To test this, we performed a membrane recruitment experiment using control and α-synuclein immunodepleted cytosol as a source of protein. After α-synuclein immunodepletion, the recruitment of AP2 to synaptic membranes was significantly reduced. These results suggest that α-synuclein participates in the recruitment of AP2 to the membrane, one of the key steps in the initiation of clathrin coated pits. Our next step will be to explore the localization of AP2 and α-synuclein, in subdomains of synaptic clusters in the lamprey giant axons. Finding the mechanism by which α-synuclein regulates clathrin-AP2 dependent synaptic vesicle endocytosis is important because it will allow us to understand the physiological function of α-synuclein in the synapse and how this function could be affected in pathological conditions, creating opportunities for the design of new therapeutic approaches.

P417/B427

Visualizing the Cellular Route of Entry of a Cystine-knot Peptide with Xfect.

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Cyclotides or cyclic cystine-knot peptides have emerged as a promising class of pharmacological ligands that modulate protein function. Interestingly, very few cyclotides have been shown to enter into cells. Here we studied the cellular behavior of EETI-II, a model acyclic cystine-knot peptide. We uncover that EETI-II is efficiently uptaken via an active endocytic pathway to early endosomes in mammalian cells, eventually accumulating in late endosomes and lysosomes. Notably, co-incubation with a cell-penetrating peptide (Xfect) enhanced the cellular uptake and altered the trafficking of EETI-II, leading to its evasion of lysosomes. Internalized EETI-II subsequently accumulates in intracellular Xfect-induced detergent-resistant membrane compartments which appear to lack characteristic endosomal or lysosomal markers. Our results demonstrate the feasibility of modulating the subcellular distribution and intracellular targeting of cystine-knot peptides, and underscore the need for the development of effective tools to enhance the cytosolic delivery of cystine-knot peptides.

P418/B428

Potential Role for Snx17 in Coupling Receptor Cargo to Endosomal Fission Machinery.

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Following endocytosis, receptors internalized to sorting endosomes (SE) are sorted to different pathways, in part by sorting nexin (SNX)proteins. Notably, SNX17 interacts with a multitude of receptors

in a sequence-specific manner to regulate their recycling. However, the mechanisms by which SNX17-labeled vesicles that contain sorted receptors bud and undergo vesicular fission from the SE remain elusive. Recent studies suggest that a dynamin-homologue, EHD1, catalyzes fission and releases endosome-derived vesicles for recycling to the plasma membrane. However, the mechanism by which EHD1 is coupled to various receptors and regulates their recycling remains unknown. Herein, we seek to characterize the mechanism by which EHD1 couples with SNX17 to regulate the recycling of SNX17-interacting receptors. *Our central hypothesis is that SNX17 couples receptors to the EHD1 fission machinery.* Co-immunoprecipitations and in vitro assays provide evidence that EHD1 and SNX17 directly interact. In addition, SNX17 and EHD1 partially co-localize on endosomes. Moreover, SNX17-containing endosomes are larger in EHD1-depleted cells compared to wild-type cells, suggesting that EHD1 depletion impairs SNX17-endosomal fission. This study helps clarify our current understanding of endocytic trafficking, by providing significant new insight into one of the least characterized trafficking steps—endosomal fission.

P419/B429

Stabilin-2 Is a Major Clearance Receptor for Bacterial Lipopolysaccharides in the Liver.

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Lipopolysaccharides (LPS) is a membrane lipid commonly associated with Gram negative bacteria and induces rapid inflammation by immune cells. The liver is the primary clearance organ of LPS in blood which commonly originates from the gut flora. Previous reports indicate that both Kupffer cells and hepatocytes bind and internalize LPS by scavenger receptor B1 (SR-B1) which has a slow internalization turnover rate. In contrast, blood-born LPS is cleared very rapidly by liver sinusoidal endothelial cells (LSECS) to prevent or mitigate a swift inflammatory response. Here, we provide evidence that Stabilin-2 is the primary scavenger receptor for LPS which undergoes rapid endocytosis and recycling within 11 minutes. LPS was modified with 4-hydroxyl-N-methylbenzimidine and radiolabeled with carrier-free ^{125}I using the Chloramine T method. The modification did not affect LPS-mediated activation of RAW264.7 macrophages. To assess specific endocytosis of LPS by the Stabilin receptors, one microgram/mL ^{125}I-LPS was incubated with HEK293 cells stably expressing either Stabilin-1 or Stabilin-2 receptors. Only Stabilin-2 cells showed robust internalization of ^{125}I-LPS over Stab1 cells and empty vector control cells that do not express any Stabilin receptor. Liver sinusoidal endothelial cells (LSECs) were also isolated and purified from mouse livers from the wildtype C57BL/6J strain and genetically modified strains as follows: Stabilin-1 KO, Stabilin-2 KO, and Stabilin-1/-2 double KO. Our preliminary results suggest that the single KO strains of the Stabilins decreased uptake of LPS and the double KO had a substantial decrease in LPS internalization. A decrease in LPS uptake was also correlated with increased cytokine production from bone marrow derived macrophages in the KO mouse lines. These data demonstrate that LPS is rapidly eliminated from circulation in part by the Stabilin receptors to mitigate activation by macrophages.

P420/B430

Clathrin Lattices Are a Dynamic Signaling Platform for the Egf Receptor in Human Cells.

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Clathrin-mediated endocytosis (CME) is key to internalize solutes, lipids, and integral proteins from the plasma membrane of eukaryotic cells. It is well known that classical CME occurs through the assembly of clathrin-coated pits which invaginate to form clathrin-coated vesicles. Additionally, clathrin can assemble as flat honey-comb like structures called flat clathrin lattices (FCLs) or plaques. FCL biogenesis and its potential roles in cell signaling, however, remains elusive. Here we used quantitative fluorescence and electron microscopic imaging to show that clathrin remodels in response to external signals such as the Epidermal Growth Factor (EGF). Specifically, nanoscale analysis of the plasma membrane show a dramatic increase in plaque-covered areas and plaque size with EGF stimulation. These effects required Epidermal Growth Factor Receptor (EGFR) interactions with EGF and β5-integrin phosphorylation by Src. Remarkably, agonist leads to persistent recruitment of EGFR, β5-integrin and Grb2 into clathrin structures and a corresponding loss of Src and Akt. Our results suggest that clathrin plaques remodel in response to growth factor signals and act as stable platforms capable of clustering and organizing dynamic signaling complexes at the plasma membrane.

P421/B431

Cargo-dependent Recruitment of the Endocytic Adaptor Protein Sla1.

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Selective internalization of integral membrane proteins from the cell surface is mediated by their interaction with endocytic machinery adaptor proteins. Emerging evidence suggests the converse relationship could also be true and, therefore, adaptor protein recruitment to the plasma membrane may depend on binding to endocytic cargo proteins. To test this idea we analyzed the yeast adaptor protein Sla1, which binds membrane proteins harboring the endocytic signal NPFxD via the Sla1 SHD1 domain. Consistent with the premise, point mutation of the SHD1 domain at key residues that disrupt binding to NPFxD caused a reduction of Sla1-GFP recruitment to endocytic sites. As previously reported, deletion of the Sla1 SR region that links Sla1 to Pan1 and End3 uncoupled Sla1-GFP from endocytic sites and resulted in broad recruitment of Sla1-GFP to the plasma membrane. Simultaneous deletion of the SR region and SHD1 point mutation resulted in total loss of Sla1-GFP localization to the plasma membrane indicating that interaction with cargo plays a key role in Sla1 recruitment. FRAP experiments with Sla1-ΔSR-GFP showed quick fluorescence recovery both at endocytic sites and at other locations consistent with a cargo-based recruitment to the plasma membrane. While expression of the SHD1 domain alone could not be detected at the plasma membrane, two and three SHD1 copies in tandem did result in plasma membrane localization suggesting multiple interactions are needed for stable recruitment. Interestingly, a Sla1 fragment containing just the SH3-3 and SHD1 domains also resulted in robust and broad plasma membrane localization. Given that SH3-3 binds ubiquitin, this result indicates the SH3-3-SHD1 fragment is recruited to the membrane via interaction with both NPFxD-containing and ubiquitinated plasma membrane proteins. Consistently, point mutation that impede SH3-3-SHD1 fragment binding to NPFxD or ubiquitin drastically reduced membrane recruitment. Our results also infer that the Sla1 SR region may regulate the interaction of Sla1 with NPFxD-containing cargo, potentially limiting Sla1 recruitment to the plasma membrane outside of endocytic sites. In addition

reduced cargo binding by Sla1 results in enhanced nuclear localization, giving new insight and support into the concept of endogenous nuclear Sla1.

P422/B432

AP-1B Facilitates Endocytosis during Cell Migration.

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The epithelial cell-specific clathrin adaptor complex AP-1B has a well-established role in polarized sorting of cargos from recycling endosomes to the basolateral membrane (Fölsch, 2015). Here we demonstrate a novel function for AP-1B during collective cell migration of epithelial sheets. We show that the cell-matrix adhesion molecule β1 integrin was dependent on AP-1B and its co-adaptor, autosomal recessive hypercholesterolemia protein (ARH), for sorting to the basolateral membrane. During cell migration, we found that AP-1B colocalized with β1 integrin in focal adhesions. Using stochastic optical reconstruction microscopy (STORM) and live total internal reflection fluorescence (TIRF) imaging we identified numerous AP-1B-coated structures at or close to the plasma membrane in cell protrusions. Using TIRF microscopy on fixed specimens we further found that AP-1B localized to the plasma membrane in areas that are distinct from AP-2 localization and both AP-1B and AP-2 did not show overlapping staining. Moreover, immuno-electron microscopy confirmed AP-1B labeling in clathrin-coated vesicles and pits originating at the plasma membrane in cell protrusions. Taken together, our data establish a novel role for AP-1B in endocytosis. Notably, we found that expression of AP-1B slowed epithelial-cell migration in wound healing assays using gain-of-function and loss-of-function approaches. Importantly, qRT-PCR analysis of human epithelial-derived cell lines revealed a loss of AP-1B expression in highly metastatic cancer cells indicating that AP-1B-facilitated endocytosis during cell migration might be an anti-cancer mechanism. Reference:Fölsch, H. 2015. Role of the epithelial cell-specific clathrin adaptor complex AP-1B in cell polarity. *Cell Logist*. 5:e1074331.

P423/B433

Ultrafast Endocytic Vesicle Trafficking at Synapses.

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Endocytosis allows a cell to remodel its membrane architecture and membrane protein composition. In most cell types, vesicles are delivered to endosomes for recycling or other processes by a stochastic interplay between active transport (molecular motor-mediated) and passive diffusion. However, trafficking mechanisms at neuronal synapses must be rapid and precise because synaptic vesicles, used for neurotransmission, must be recycled in seconds. Traditionally, synaptic vesicle recycling was thought to involve endocytosis alone, but recent work suggests a two-step system, whereby endocytic vesicles are trafficked to a synaptic endosome for synaptic vesicle regeneration. Strikingly, both endocytosis and endosomal trafficking occur within 1 s following fusion —the kinetics and precision of which cannot be explained by classic transport mechanisms (1-2 minutes). Thus, it is unclear how this ultrafast trafficking occurs at synapses. My preliminary data suggest that actin polymerization and Intersectin-1, a multimodal endocytic adaptor protein implicated in various synaptic functions, are required for the kinetics of post-endocytic trafficking of endocytic vesicles. However, how Itsn1 activity is regulated, what actin-related factors Itsn1 works with, and how nascent actin polymers function during post-endocytic trafficking remains unclear.

P424/B434

Regulation of Actin Polymerization during Clathrin-mediated Endocytosis by the Tda2-Aim21 Complex.

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Clathrin-mediated endocytosis (CME) is a major endocytic pathway that is essential in all eukaryotic cells. The highly conserved process involves the sequential assembly of nearly 60 proteins at endocytic patches along the plasma membrane. These endocytic factors work together to collect cargo into a clathrin-coated pit, invaginate the surrounding membrane, and pinch off the invagination as a vesicle inside the cell. In budding yeast CME, polymerization of actin into a branched network is critical to provide the force necessary for membrane invagination. Recently, our lab identified Tda2 as a novel component of the CME machinery. Unexpectedly, the crystal structure of Tda2 revealed it to be a homodimer and structural homolog to the TcTex1 type dynein light chain, the first of its kind in yeast. Additionally, Tda2 was found to form a stable complex with the little-studied endocytic factor Aim21. Despite these findings, the function of Tda2 in CME has remained largely elusive. Here, we utilized numerous biochemistry and cell biology-based techniques to further elucidate the molecular function of Tda2 during CME in budding yeast. The interaction of Tda2 to Aim21 was mapped to a 16-amino acid sequence of Aim21 near its C-terminus. Two amino acids of Aim21, F533 and W536, were found to be particularly important for the interaction with Tda2. Fluorescence microscopy showed that these hydrophobic amino acids are critical for the localization and function of Tda2. Biochemical techniques demonstrated that the Tda2 homodimer functioned to bring together two copies of Aim21, acting as a dimerization chaperone. In turn, dimerization of Aim21 near its C-terminus proved necessary for its interaction with Actin Capping Protein (Cap1/2), an endocytic factor that binds the barbed-end of actin filaments, preventing addition or loss of actin subunits. Disruption of the Tda2-Aim21 interaction resulted in decreased levels of Cap1/2 at endocytic patches and a prolonged phase of actin polymerization. Thus, this work has shown Tda2 to function as a dimerization chaperone for Aim21 during CME, supporting what others have postulated as the function of dynein light chains in the dynein motor complex. Moreover, the Tda2-Aim21 complex through its interaction with Cap1/2 appears to be an important player in regulating the branched actin network during yeast CME.

P425/B435

α-Arrestin Regulation of Protein Trafficking: Using Evolutionary Rate Covariation to Define Protein Trafficking Regulatory Networks.

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Alpha-arrestins help cells survive environmental changes by controlling membrane protein trafficking. One hurdle to understanding α-arrestins is that few α-arrestin-cargo pairs have been identified. It is technically challenging to identify membrane cargos due to their transient associations with α-arrestins and their biochemical nature. to identify α-arrestin-regulated cargos, we used Evolutionary Rate Covariation (ERC), which employs sequence-based signatures to identify genes with similar evolutionary histories. We compared ERC values for α-arrestins with cargos across 18 yeast species. Among the top co-evolving proteins were those previously defined as α-arrestin cargos. We are determining if the membrane proteins with the highest ERC values (>0.5) are α-arrestin cargos by assessing their localization and relative protein abundances in wild-type cells versus those lacking the specific α-arrestins. Fluorescence intensity was used to quantify the abundance and/or subcellular distribution of

GFP tagged proteins. Statistically significant changes in GFP abundance or localization between wild-type cells and those lacking α-arrestins demonstrate dependence on these protein trafficking adaptors. This makes them good candidates as new α-arrestin-dependent cargos, which we are confirming by co-localization, bimolecular fluorescence complementation and biochemical approaches. Using ERC and our fluorescence imaging pipeline, we have quantitatively confirmed that 36 integral membrane proteins previously unassociated with α-arrestins display α-arrestin-dependent localization changes. This greatly expands the repertoire of α-arrestin cargo within cells, and raises important functional implications for this family. In conclusion, the ERC approach is a powerful new tool that is able to define protein trafficking regulatory networks, which will undoubtedly be of interest to the cell biology community.

P426/B436

Studying Dynamics and Mechanics of Clathrin-mediated Endocytosis in the Native Tissue Context.

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Clathrin-mediated endocytosis (CME), an essential cellular function in eukaryotes, has been extensively studied using adherent cultured cells. These cells, however, are artificially flat and lack the physiological tissue environment, which influences the mechanical properties of the plasma membrane. Thus, the question how CME is adapted to the functional and architectural diversity of cells and tissues, remains poorly understood. Here, we aim to understand how CME functions in the native tissue context. Generally, we ask whether CME is performed by an adapted machinery and occurs with specific spatiotemporal dynamics in different cells and tissues. We used *Drosophila melanogaster,* which combines the advantages of a versatile genetic toolkit, a comparably small genome and accessibility to a variety of imaging modalities. Using CRISPR we created fly lines where endocytic proteins are endogenously tagged with fluorescent markers. We then used fluorescence microscopy and particle tracking to follow individual endocytic events and measure their dynamics with high spatiotemporal resolution.In our experiments, we imaged intact *Drosophila* pupae, which are non-motile. We focused on the single-layered epithelium at the notum, which is at the very surface and thus well-suited for microscopy. Within this epithelium, individual precursor cells undergo stereotypic divisions to form mechanosensory organs, each consisting of a bristle, socket, neuron and sheath cell. We use this organ as model system to image CME within tissue, throughout cell division and differentiation. This approach allowed us to record individual endocytic events in living fly pupae with a similarly high resolution as in cell culture, allowing us to precisely determine their lifetimes and dynamic trajectories. We find that endocytic events are temporally highly regular, and observed intriguing changes in the dynamics of endocytic events in bristle cells. We hypothesize that in these cells, CME is reprogrammed during development, potentially in response to changing cellular function and architecture. To investigate this question in more detail, we will study how actin and actin-binding proteins contribute to force generation during CME, how changes in membrane tension affect endocytic dynamics, and how CME adapts during differentiation also in the neuron and the socket cell of the mechanosensory organ.

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Protein Droplets Catalyze Assembly of Endocytic Vesicles.

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During clathrin-mediated endocytosis, dozens of adaptor and coat proteins assemble into a mesh-like network at the plasma membrane, where they work together to drive vesicle formation. Fcho1 and Eps15 are among the earliest adaptors to arrive at endocytic sites. These initiator proteins are responsible for recruiting, concentrating and organizing downstream components at the membrane surface. However, as the budding vesicle grows, the initiator proteins must also allow dynamic rearrangement of the adaptor network. It is unclear how initiator proteins are able to meet these competing demands. Toward explaining this apparent paradox, we have made the exciting discovery that Fcho1 and Eps15 assemble into liquid protein droplets at membrane surfaces. Droplet assembly requires specific protein domains in Fcho1 and Eps15 that contribute to the ability of the two proteins to form a multivalent network. Further, these droplets exhibited liquid-like properties, including coalescence and dynamic exchange of proteins in and out of droplets. Toward characterizing the phase behavior of these initiators over a range of biochemical conditions, we have modulated protein concentrations, temperature, and crowding conditions to develop a set of phase diagrams. The liquid properties of Fcho1/Eps15 protein droplets can explain how the downstream adaptor network is tightly concentrated yet also dynamic. To test the role of Fcho1/Eps15 liquid assemblies in live mammalian cells, we developed an assay that allows us to tune the strength of initiator protein assembly in real time using light. Specifically, we created a chimeric version of Eps15 that contains a light-inducible oligomerizing domain, Cry2. Application of low light levels drove fluid assembly of the initiator proteins. These fluid assemblies resulted in an increase in endocytic initiation events. In contrast, high light levels drove the formation of solid initiator complexes, which stalled vesicle dynamics. These results indicate that solid assemblies of initiator proteins are unable to release endocytic assemblies. In contrast, more fluid assemblies can serve as strong yet dynamic catalysts. These results suggest that the liquid-like behavior of Fcho1/Eps15 droplets is important for effective catalysis of clathrin-mediated endocytosis in cells.

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CMEpi, a Potent and Selective Structure-based Inhibitor of Clathrin-mediated Endocytosis.

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Clathrin-mediated endocytosis (CME) is a predominant route of entry into cells via the formation of clathrin-coated vesicles. The major coat proteins are clathrin and AP2. Tools to address CME function, especially a specific inhibitor that works by rapid and well-defined mechanism, are in great demand. Here we revealed that overexpression of N-terminal fragments of the clathrin heavy chain encoding the terminal domain (TD) with or without the distal leg potently and specifically inhibit CME and CCP dynamics, primarily through the interference of AP2 and SNX9 functions. Furthermore, we designed small membrane-penetrating peptide mimics of the binding surfaces on the TD and observed differential inhibitory abilities. A peptide corresponding to the W-box motif binding site was identified as the strongest CME inhibitor, also by the mechanism of inhibiting AP2 and SNX9 functions. This peptide

inhibitor, which we named CMEpi, does not affect other endocytic pathways and even other clathrin-dependent trafficking event, such as AP1-dependent Golgi trafficking.

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Regulation of the Yeast Transporter-like Glucose Receptor-mediated Signaling Pathway.

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Defects in glucose sensing are at the root of a number of metabolic disorders. Impaired regulation of blood glucose levels may cause severe metabolic disorders, such as diabetes, and many types of cancer cells depend on a high rate of glucose consumption to maintain their viability. Thus, learning how cells sense and respond to glucose is of great interest and major significance. The yeast *Saccharomyces cerevisiae* senses glucose through two transporter-like glucose receptors (TLGRs) Rgt2 and Snf3, formerly known as glucose sensors. Evidence indicates that TLGRs act as glucose receptors that generate an intracellular signal in response to glucose that induces expression of genes involved in glucose uptake and metabolism. However, the underlying mechanisms are largely unknown. The objective of this study is to test the hypothesis that TLGR signaling activity may be regulated by extracellular glucose concentrations. To achieve this objective, we assessed the cell surface expression of TLGRs using biochemical and cell biological approaches. Several lines of evidence show that TLGRs are endocytosed and degraded in the vacuole in glucose-starved yeast cells and that TLGR turnover is mediated by ubiquitination. TLGRs are mainly localized to the plasma membrane when glucose is present but targeted to the vacuole for degradation when glucose is depleted from the medium. However, TLGR turnover is impaired in cells lacking the End3 protein involved in endocytosis, the Doa4 deubiquitinase and the Rsp5 ligase. It has been postulated that Rgt2 and Snf3 have respectively low and high affinities for glucose. Our results show that Rgt2 is endocytosed and degraded in low glucose-grown cells, whereas Snf3 is downregulated in cells grown in high glucose. Thus, Rg2 is expressed at the cell surface in low conditions, while Snf3 is expressed in high glucose conditions. Consistently, constitutively active TLGRs $Rgt2^{R231K}$ and $Snf3^{R229K}$ are shown not to undergo endocytosis, suggesting that both are endocytosis resistant TLGRs. Taken together, our results suggest that cell surface expression levels of TLGRs are tightly associated with their ability to sense glucose. In conclusion, it has long been hypothesized that TLGRs are expressed constitutively and undergo a conformational change upon glucose binding, leading to activation of the receptors. By contrast, our results show that TLGRs are constantly targeted to the vacuole for degradation and expressed at the cell surface only when the extracellular concentration of glucose is in the range of their respective affinities and suggest a novel mechanism for this glucose sensing and signaling system.

P430/B440

Rock2 Mediated Myosin Contractility Is Required for Internalization of CIE Cargo Proteins.

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Endocytosis is an important event that cells utilize to internalize cell surface proteins and fluid into the cell. There are two main forms of endocytosis: clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE), amongst which CME is the most studied. While CME is important for the internalization of specific surface proteins, bulk trafficking of fluid and membrane occurs primarily in a clathrin-independent manner. While we have established the trafficking itinerary of many classical CIE cargoe proteins, such as CD59 and Major Histocompatibility Complex Class I (MHCI), little is known

about the cellular machinery involved in CIE. To identify proteins essential for the internalization of the GPI-anchor protein CD59 and the cell surface protein MHCI in Hela cells, we designed an siRNA screen utilizing the Dharmacon™ Membrane Trafficking library, which contains siRNA targeting 140 established membrane trafficking genes. Among these candidates was the kinase ROCK2, which upon depletion inhibited internalization of both cargo proteins, a result which was further confirmed using small molecule inhibitors of ROCK2. This phenotype was specific to ROCK2, as knockdown of the homologous protein ROCK1 had no effect on the internalization of either cargo. ROCK2 regulates the phosphorylation and subsequent deactivation of the actin severing protein Cofilin. Knockdown of Cofilin stimulated the internalization of both MHCI and CD59 and resulted in the accumulation of cargo in enlarged vesicles located in a dense actin network at the cell periphery. ROCK2 also positively regulates myosin contractility through the phosphorylation and subsequent activation of myosin light chain (MLC). Inactivation of Myosin II using blebbistatin results in decreased internalization of both cargo, to a similar extent as ROCK2 knockdown. This would suggest that ROCK2 regulation of both MLC and Cofilin is important for CIE cargo internalization. To further characterize Myosin II in CIE, two isoforms of Myosin II (Myo2A and Myo2B) were inhibited and CIE was monitored. Interestingly, internalization of MHCI required Myo2A, while CD59 required Myo2B. There is evidence to suggest that in polarized cells distinct functions of Myo2A and Myo2B are more evident. In polarized Caco-2 cells, we observe that CD59 localizes and internalizes via the apical domain and MHCI internalizes via the basolateral domain. Silencing of Myo2A in polarized Caco-2 cells inhibits MHCI internalization, while inhibition of Myo2B has no effect. This would suggest that CIE in polarized cells also exhibits preferential myosin isoform requirements, and makes polarized cells an intriguing system for gaining insight into differential Myosin II isoform requirements for internalization of CIE cargo proteins.

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CLIC Cargo Uptake Is Largely Dependent Upon Microtubule Integrity and Microtubule-Associated Motor Functions.

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Cellular internalization pathways can be categorized, based on their requirement for clathrin components, into clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE). One major pathway of the CIE group is the clathrin-independent carrier (CLIC) or glycosylphosphotidylinositol-anchored protein enriched compartments (GEECs) mediated endocytosis. CLIC/GEEC is responsible for the internalization of many physiologically important cell surface factors and channels including CD44, CD59, CD147, and MHCI. Yet the mechanisms of CLIC cargo uptake is not very well understood. In recent years, accumulated evidence from our lab and other groups suggests the microtubule network and dynein motors are closely linked to this process, perhaps by facilitating membrane fission and the generation of CLIC/GEEC carriers. Utilizing cell surface receptors CD44 and CD59, we seek to further elucidate the relationship between CLIC uptake, the microtubule network, and dynein motors. We demonstrate that compared to the uptake of transferrin by CME, CD44 and CD59 not only internalize through distinct compartments and components, but CLIC uptake is far more sensitive to microtubule disruption by the drug Nocodazole, suggesting a higher dependency on the integrity of microtubule network. Furthermore, we show that in comparison to CME, the CLIC/GEEC pathway displays higher sensitivity to dynein motor inhibitor Ciliobrevin D, as well as to the overexpression of p50/dynamitin, which acts as a dominant negative construct for the dynein complex. However, we observe the effects of dynein motor inhibition on CLIC uptake is less dramatic than that of

the microtubule disruption, possibly hinting at the involvement of other motors and/or alternative mechanisms. Our data contribute to a growing body of evidence suggesting a complex network of microtubule and motor components collaborating to bring in CLIC cargos, in a mechanism remarkably different from the well-established CME pathway.

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Phase Separation in the Early Endocytic Protein Network.
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Clathrin-mediated endocytosis is a major pathway used by eukaryotic cells to produce transport vesicles from the plasma membrane. In yeast, the assembly of endocytic machinery is an ordered process that involves dozens of different proteins such as clathrin adaptors, coat proteins and actin cytoskeletal proteins. The initiation step of endocytosis is highly redundant, but the exact mechanism that underlies this flexibility is unknown. The early coat is a dynamic network of weak protein-protein interactions. Many early proteins have high turnover rates, and yet their function is to recruit and concentrate cargo and downstream effectors. Ede1, the yeast homologue of mammalian Eps15, is one of the earliest-arriving endocytic proteins. In EDE1 deletion strains, many early proteins become diffuse on the membrane instead of assembling into discrete endocytic sites. This indicates that Ede1 is a key factor in early site formation. However, Ede1 lacks membrane-binding domains and relies on ubiquitinylated cargo and endocytic adaptors for recruitment to the PM. In strains deficient in such adaptors, the Ede1 protein forms bright and long-lived clusters. We show that this phenotype is concentration-dependent and can be mimicked by over-expressing Ede1. We also demonstrate that that these clusters exhibit dynamic properties of liquid droplets and that temperature can modulate their phase behaviour. The droplets also recruit many other endocytic proteins, and can initiate bursts of actin polymerization. In many respects, the droplets resemble genuine endocytic sites. We used the droplet formation as a tool to study the properties of Ede1 and discovered that the phase separation depends on a core region containing a low-complexity sequence and a coiled-coil domain. When we deleted this core region, Ede1 and other early proteins no longer localized to distinct endocytic sites, and initiation was impaired. On the other hand, fusion with the Ede1 core region induced a punctate localization of an otherwise diffuse heterologous membrane associated protein. These results allow us to explain the importance of self-associating Ede1 regions in endocytic initiation. We also propose that the concentration of cargo and adaptors during the early phase of endocytosis might be a phase separation process facilitated primarily by Ede1.

Rab GTPases

P433/B443
Arf-like Gtpase 15 Modulates the Biogenesis of Filopodia.
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Arf and Arf-like (Arl) GTPases regulate several cellular processes such as intracellular transport, cytoskeletal organization etc. The functions of few Arls have been well studied, but the role of Arl15 in any of these processes remains elusive. Studies have reported that Arl15 may influence the adiponectin levels and is also implicated in metabolic disorders like type 2 diabetes, coronary heart disease, rheumatoid arthritis and childhood obesity. Further, Arl15 is shown to associate with golgi upon insulin

stimulation. In this study, we aim to understand the molecular function of Arl15 in vesicular transport and other cellular processes by using cellular model system. Expression of Arl15 as a GFP/mCherry fusion in several cell types revealed its localization to cell surface, protrusions and tunnel-like nanotubes (TNTs). Consistently, Arl15 in HeLa cells colocalize with PLCdelta-PH (PIP2 binding protein), Fascin (filopodia-specific protein), phalloidin (labels F-actin) and M-Sec (TNT marker). Intracellularly, Arl15 localizes to golgi and a cohort to endocytic-recycling compartments (ERCs). As expected, Arl15 showed colocalization with Rab11/22A (ERC markers) and GM130/Golgin 245 (golgi-tethering proteins). To understand the dynamic association of Arl15 with multiple membranes, several point mutations were carried out as identified in homology analysis with other Arl family members. These mutations include $Arl15^{S2G}$ (myristoylation-positive mutant), $Arl15^{S2A}$ (phospho-dead mutant), $Arl15^{S2E}$ (phospho-mimetic mutant), $Arl15^{T46N}$ (dominant negative mutant), $Arl15^{A86L/A86Q}$ (constitutive active mutant) and $Arl15^{S2G, A86L}$/$Arl15^{S2G, T46N}$ (double mutants). Interestingly, $Arl15^{A86L}$ and $Arl15^{S2G, A86L}$ localize to the cytosol and disengage from the cell surface/filopodia, suggesting $Arl15^{A86L}$ possibly acts as a dominant negative mutant. Next, we tested the role of cortical actin in recruiting Arl15 to the filopodia. Latrunculin a treatment did not alter the cell surface/golgi localization of Arl15. However, golgi fragmentation by using brefeldin-A (targets GBF1, Arf1 GEF) completely abolished the Arl15 localization, similar to Arf1 and other golgi proteins like Grasp-55. These results indicate the Arf1-dependency for Arl15 recruitment to the membranes. In line, over expression of dominant negative mutant of Arf1, $Arf1^{T31N}$ mislocalizes Arl15 to cytosol, suggesting Arf1 GTPase cycle is crucial for the Arl15 localization. Preliminary analysis of Arl15-knockdown in HeLa cells showed defective cell migration and transferrin recycling without affecting the golgi integrity. Overall, this study indicates that Arf1 dependent recruitment of Arl15 possibly regulates the vesicular transport from golgi towards the cell surface for filopodia biogenesis.

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Tumor Suppressor Activity of Rab17 Is GTP-dependent.

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We recently found that rab17 regulates the last step of hepatic basolateral-to-apical transcytosis via selective interactions with syntaxin-2 at the apical surface. We further determined that optimal apical vesicle docking/fusion requires rab17 mono-sumoylation and GTP hydrolysis. Studies by others have implicated rab17 as a tumor suppressor in hepatocellular carcinoma (HCC) based on the finding that patient malignant lesions expressed significantly lower levels of rab17 compared with adjacent paraneoplastic tissue. Furthermore, rab17 overexpression in HCC cell lines ameliorated the tumorigenic phenotype while its knockdown enhanced invasiveness. We are examining the mechanisms by which rab17 antagonizes the oncogenic phenotype. Because rab17 overexpression promotes dendrite formation in neuronal cells, we have been monitoring actin-based surface structures in hepatoma cells exogenously expressing wild-type rab17, sumoylation-deficient (K68R), or constitutively GTP- (Q77L) or GDP-bound (N132I) rab17. Consistent with studies in neuronal cells, expression of wild-type rab17 cells led to the formation of filipodia-like protrusions and cell ruffles with rab17 colocalized with syntaxin-2 at the distal tips and in ruffles. Interestingly, expression of either the GTP-bound or sumo-deficient rab17 promoted modest membrane ruffling, blunted protrusion formation and maintained colocalization with syntaxin-2. However, expression of wild type, GTP-bound and sumo-deficient rab17 did not change the surface distributions of Mena, VASP or WDR1. In contrast, GDP-bound rab17 did not distribute to the cell surface nor did it promote changes in cell morphology, but it did lead to loss of VASP cell surface distributions. Despite the formation of actin-based protrusions, cells expressing either wild-type or GTP-

bound rab17 showed impaired migration when monitored by live cell imaging in scratch assays whereas cells expressing GDP-bound rab17 displayed no changes in migration. These results suggest that rab17 expression promotes GTP-dependent protrusion formation which correlates with impaired motility. Based on rab17 colocalization with syntaxin-2, we propose that rab17 antagonizes the oncogenic phenotype by redirecting vesicular cargo to distinct surface domains thereby preventing cellular reorganization required for collective cell migration and invasion.

P435/B445

An Enlarged Rab4-modulated Endosomal Compartment Is the Site of Prolonged Egfr Activation in Breast Cancer Cells.

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Early sorting endosomes are responsible for the proper trafficking and function of membrane-bound receptors, required for iron uptake or signaling regulation, such as transferrin receptor (TfR) and epidermal growth factor receptor (EGFR), respectively. However, the morphology, receptor composition, and signaling of early endosomes remain poorly understood in breast cancer. We have identified a novel population of enlarged early endosomes in breast cancer cells and human tumor xenografts (human breast cancer MDAMB231 and T47D cells) but not in noncancerous epithelial cells (MCF10A). Quantitative analysis of endosomal morphology, cargo sorting, EGFR activation and Rab-GTPase regulation were performed using super-resolution and confocal microscopy followed by 3D rendering image analysis using Imaris software. Results show that aggressive triple-negative MDAMB231 cells have significantly fewer, but much larger EEA1-positive early endosomes compared to MCF10A cells. These large endosomes contain adjacent Rab4 and Rab11 domains and undergo cargo recycling via budding from actin microdomains. Live-cell imaging indicated that endocytic cargo, e.g. EGF and Tf, traffic together via these enlarged endosomes in MDAMB231 cells, but not in MCF10A. Most importantly, these large EEA1-positive MDAMB231 endosomes exhibited prolonged and increased EGF-induced activation of EGFR (p1068) using cell imaging and morphometric analysis. Importantly, knockdown of Rab4 in MDAMB231 cells lead to increased EGF-induced activation of EGFR (p1068), without significant effect on MDAMB231's already large endosomal size. In contrast, Rab4 knockdown increased endosome size but did not affect EGFR signaling activation in MCF10A cells. These results suggest that Rab4 affects endosome size and EGFR signaling differently in breast cancer vs. non-cancerous epithelial cells. Moreover, these results make evident the importance of determining the role of endosomal morphology in the regulation of EGFR signaling in breast cancer cells. In summary, an extensive characterization of early-endosomes in breast cancer cells has identified a Rab4-modulated enlarged endosomal compartment as the site of prolonged and increased EGFR activation. These findings establish the early endosomes as central players in the regulation of cargo trafficking and cell signaling in breast cancer cells.

P436/B446

ALS2 Controls the Intracellular Localization of Small GTPase Rab17 and Regulates Endosome Maturation.

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ALS2, an autosomal recessive form of childhood-onset amyotrophic lateral sclerosis (ALS), is caused by loss-of-function mutations in the *ALS2* gene. The ALS2 protein (ALS2), the product of this gene, acts as a guanine nucleotide exchange factor (GEF) for small GTPase Rab5 and regulates the fusion and maturation of early endosomes in cells. Since ALS2 is relocalized from the cytosol to endosome by macropinocytosis via Rac1-induced membrane ruffles, it has been surmised that loss of ALS2 function results in dysfunctional intracellular membrane trafficking. However, the molecular mechanisms underlying the pathogenesis of ALS2-linked recessive motor neuron diseases are still unclear. In this study, to gain further insight into the ALS2-associated cellular functions, we focused on a novel ALS2-interacting small GTPase Rab17 and investigated the functional relationship between ALS2 and Rab17. First, we examined whether ALS2 could bind to Rab17 and act as a GEF for Rab17. GST pull-down assay revealed that ALS2 preferentially bound to $Rab17^{WT}$ (WT: wild type) and $Rab17^{CN}$ (CN: constitutively negative). However, no observable ALS2-mediated GEF activity on Rab17 was detected by the GDP-dissociation assay. The results suggest that ALS2 does not function as a GEF, but rather acts as a modulator for Rab17. Next, to clarify the Rab17-linked ALS2 cellular function, we observed the intracellular localization of ALS2 and Rab17 in HeLa cells. While Rab17 was mainly localized to Rab11-positive recycling endosomes, it was also colocalized with ALS2 in early endosomal compartments under normal cultured conditions. Further, upon Rac1 activation, Rab17 was relocalized to Rac1-induced membrane ruffles followed by internalization to early endosomes like was ALS2. Remarkably, although Rab17 recruitment to endosomes was not affected by siRNA-mediated *ALS2*-knockdown (KD), loss of ALS2 resulted in enlargement of Rab17-residing endosomes, onto which Rac1, VPS35 and LAMP-1/2 but not EEA1 were colocalized. On the other hand, pulse-chase assay using Alexa594-Tfn or Alexa488-EGF revealed that, under ALS2-deficient conditions, neither Tfn nor EGF was localized to enlarged Rab17-residing endosomes, suggesting that clathrin-mediated endocytosis (CME) was unlikely to account for Rab17 internalization to endosomes. These results suggest that ALS2 regulates the trafficking of Rab17-residing endosomes, which are formed by Rac1 activation, in a CME-independent manner. In conclusion, ALS2 might play a key role in endosome maturation not only by the activation of Rab5 but also by the regulation of Rab17 molecular function, possibly through their interaction, in cells. We are currently investigating the molecular mechanism by which Rab17-residing endosomes can be enlarged in the absence of ALS2.

P437/B447

Tnf Alpha Induced Migration and Integrin Beta 3 Levels Are Regulated by the Rab Endocytic Pathway in Reactive Astrocytes.

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Introduction: Upon injury to the central nervous system, astrocytes generate a pro-inflammatory environment required for neuronal regeneration, and undergo a process called "Gliosis" characterized by hypertrophy, astrocyte migration and increased expression of proteins including glial fibrillar acidic protein (GFAP), syndecan-4 (SDC4), and $\beta_3$integrin. Our previous data indicate that the neuronal protein Thy-1 binds to $\beta_3$integrin to promote cell migration only when astrocytes are reactive. However, the mechanisms implicated in the up regulation of $\beta_3$integrin in astrocytes under pro-inflammatory conditions remain unclear. **Material and Methods:** We performed an in *silico* comparative meta-analysis of gene expression profiles obtained from publicly available microarray datasets of non-reactive vs. reactive astrocytes.Hierarchical clustering comparisons were performed using the MultiExperiment Viewer software and affected signaling pathways were examined with Kyoto Encyclopedia of Genes and Genomes Database. DITNC1 astrocytes were treated with TNF-α for 48hrs to induce reactivity in *vitro and* $\beta_3$integrin levels were measured by Western blot and flow cytometry. GTPase activities were measured using pull-down assays. Astrocyte migration was induced using Thy-1 as a stimulus and evaluated in transwell assays. Results were compared with unpaired t tests of at least three independent experiments with a p value ≤0.05 for significance. **Results: in** the in *silico* comparative meta-analysis we found that Rab endocytic pathways were altered in reactive astrocytes. TNFα treatment changed protein expression associated with the appearance of reactivity markers, such as GFAP, and increased surface levels of $\beta_3$integrin in the DITNC1 cells. This same treatment altered Rabs and Rac1 GTP loading. Thy-1-induced astrocyte migration was also associated with the changes observed in Rab protein levels. **Conclusions:** These studies aid in understanding the molecular mechanisms involving Rab-dependent pathways in astrocyte activation, an important process observed in pathological conditions, such as neurodegenerative diseases e.g.Amyotrophic Lateral Sclerosis and brain injury.

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The Amphipathic Motifs of Some Arf Proteins Are Required and Sufficient for Determining Specific Interacellular Localizations.

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The localizations of Arf family proteins are generally thought to be determined by their corresponding guanine nucleotide exchange factors (GEFs). However, although the GEFs directly interact with the switch domains of Arf proteins, the amphipathic motifs at the N-terminus of Arf proteins are also important for their membrane associations. Here, we analyzed the functional roles of the amphipathic motifs of the Golgi-localized Arf family protein, Arfrp1, the endoplasmic reticulum-localized Arf family protein, Sar1A, and the endosome- and plasma membrane-localized Arf family protein, Arl14. Switching the amphipathic helix motifs among these Arf proteins causes changes of their intracellular localizations. Moreover, we found that the amphipathic helices of Arfrp1, Arl14 and Sar1A are sufficient to bring cytosolic proteins to the Golgi, the endosomes or the ER respectively. The spatial determination

mediated by Arfrp1 helix requires its binding partner Sys1. In addition, the acetylation modification on Arfrp1 helix and the myristoylation modification on Arl14 helix are important for the spatial determination. Interestingly, Sar1A, Arfrp1 and Arl14 are recruited to their specific intracellular compartments independent of GTP binding, suggesting their GTP binding and membrane association are uncoupled. In sum, our results demonstrate that the amphipathic motifs of Arfrp1, Arl14 and Sar1A are required and sufficient for determining specific intracellular localizations. In addition, our study provides novel short motifs that can be used to target cytosolic proteins to specific intracellular compartments.

P439/B449

Regulation of Cell Migration and GTPase Function through Mechanical Control of RNA Localization.

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Local RNA translation is emerging as a mechanism required to stabilize protrusions and promote persistence and directionality during cell movement. Our work has shown that numerous RNAs are enriched in protrusive regions, and that localization of a subset of them is promoted by increased stiffness of the extracellular matrix. The localization of this group of RNAs is important for efficient cell migration. We have now developed a method to disrupt the localization of specific individual RNAs from this group, including that encoding Rab13, a GTPase involved in vesicle trafficking. Strikingly, preventing the localization of the Rab13 RNA at protrusions is sufficient to disrupt the ability of cancer cells to migrate on 2D surfaces or to invade through Matrigel. Through biochemical and live cell imaging approaches we find that the Rab13 RNA is similarly translated in both the cell body as well as in peripheral protrusions. Interestingly, preventing the targeting of endogenous Rab13 RNA at protrusions leads to the production of less active Rab13 protein. Similar conclusions are reached when exogenous Rab13 is expressed from a mislocalized transcript. These results reveal a novel mechanism controlling Rab13 GTPase function. Specifically, they suggest that translation of GTPases in particular local environments is important for their proper activation and function during cell movement. We are further testing this hypothesis and investigating in detail the underlying mechanisms.

P440/B450

Rab39 and Klp98a Are Required for Furrow Formation during Early *Drosophila* Embryogenesis.

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The formation of a plasma membrane furrow is an essential process during development. Furrow formation is necessary for successful cell division and cytokinesis in addition to the ability to create multicellular tissues. Here we explore the role of the Golgi-associated Rab protein Rab39 in furrow formation during early *Drosophila* embryogenesis. Rab39 is one of eight Rab proteins that has been shown to localize to discrete puncta by live imaging in a screen in early *Drosophila* embryos, but its function and pathway have not been well characterized. We demonstrate that Rab39 forms dynamic, tubular structures that colocalize with trans-Golgi markers and that the knockdown of Rab39 using RNA interference causes defects in furrow length and nuclear division during syncytial cycles 10-13. Klp98A, a kinesin 3 family motor protein, produces similar abnormalities when disrupted and colocalizes with Rab39. In the absence of Rab39, Klp98A forms large complexes and accumulates at the Golgi. Additionally, Rab39 and Klp98A dynamics are dependent on microtubule, but not filamentous actin, networks. The Golgi associated tether Golgin245 also shows a strong furrow phenotype in disrupted

embryos and is another potential effector of Rab39. Together, these proteins could represent a novel pathway that mediate membrane trafficking from the Golgi to the plasma membrane to aid in furrow formation.

P441/B451

Role of Rab4 Isoforms in the Regulation of Endocytic Trafficking in Breast Cancer Cells.

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Cell surface receptors such as the epidermal growth factor receptor (EGFR) and Transferrin Receptor (TfR) are transported from the plasma membrane to the early endosome utilizing clathrin-mediated endocytosis. Rabs are a family of GTPases that control membrane identity, vesicle motility, and proper vesicular trafficking. Rab4, found on early endosomes, ensures that EGFR and TfR are properly sorted and recycled to the cell surface or the lysosome for degradation. However, in cancer cells, the endocytic pathway is highly dysregulated. Our previous work using confocal microscopy followed by 3D rendering software IMARIS demonstrated that endosomes derived from an aggressive triple-negative breast cancer cell line (MDA-MB231) were larger than endosomes observed in noncancerous epithelial cells (MCF10A). These enlarged endosomes showed prolonged and amplified EGF-mediated EGFR activation. Furthermore, Rab4A depletion in MCF10A cells demonstrated similarly enlarged endosomes but did not exhibit increased EGF-mediated signal activation. The purpose of this investigation is to study the role of Rab4, specifically isoforms a and B, on the role of endocytic trafficking of EGFR and TfR in breast cancer cell proliferation, migration, and invasiveness. Current progress in this investigation using a cell line panel real-time polymerase chain reaction (RT-PCR) assay revealed that compared to MCF10A, MDA-MB231 has increased expression of Rab 4B mRNA and decreased expression of Rab 4A mRNA. Similarly, a Luminal a breast cancer cell line (T47D) has increased expression of Rab 4B and decreased expression of Rab 4A mRNA. Compared to the MCF10A cell line, a Luminal-HER2+ cell line (AU565) and a triple-negative breast cancer cell line (MDA-MB 436) has low expression of both Rab 4A and Rab 4B mRNA levels. Future directions for this investigation will involve generating single and dual knockdowns of Rab 4A/B to assess their effect on endosome size, EGFR activation, and associated cell proliferation and invasiveness in a variety of human breast cancer cells (see above). Repeat studies and analyses will be conducted with individual and dual overexpression of isoforms Rab4A/B. By assessing the effect of Rab4 isoforms on the endocytic pathway, the role of endosomal morphology and function in breast cancer can be further elucidated. Furthermore, future advancements can be made towards understanding the process by which cancer cells hijack the endocytic pathway to proliferate and survive especially in unconducive environments. Moreover, understanding the endocytic pathway can provide insights as to why some cancer cells respond to receptor-mediated targeted therapy while others do not respond as well.

P442/B452

Expanding the Realm of Small GTPase Function: Evidence for Rab40b/Cul5 Mediated Rap2 Regulation during Cell Migration.

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One of the most fundamental questions in cell biology is how cells migrate through three-dimensional (3D) spaces. A defining feature of cell migration is the formation of actin-rich protrusions at the leading edge that are necessary for remodeling of the extracellular matrix (ECM). Extension of these actin-rich

protrusions, known as invadopodia, combined with the targeted secretion of matrix metalloproteinases (MMPs) facilitate degradation of the ECM, allowing cells to migrate and invade through their surrounding environment. However, what remains poorly understood is how cells coordinate MMP secretion and actin polymerization during the formation and extension of invadopodia. Our lab has identified Rab40b as a regulator of invadopodia formation, targeted MMP secretion, and cell migration. Rab40b is unlike any other small GTPase because in addition to the canonical Rab domain, it also contains a Suppressor of Cytokine Signaling (SOCS) domain, a binding motif known to act as a bridge between E3 Ubiquitin (Ub) Ligases and protein substrates. In this study we demonstrate that Rab40b binds to Cullin5 (Cul5), a known E3 Ub Ligase module critical for protein ubiquitylation and degradation. We have also identified Rap2 as a putative substrate of the mammalian Rab40b/Cul5 complex. Importantly, the Rap family of small GTPases, including Rap2, have been linked to the regulation of cell-cell adhesions and actin cytoskeleton dynamics. Here we show that Rab40b/Cul5 binds and regulates Rap2 by mediating its mono-ubiquitylation. Interestingly, we demonstrate that inhibition of Rap2 mono-ubiquitylation leads to endocytic removal from the plasma membrane and rapid lysosomal degradation, thus, terminating Rap2 signaling. Based on our preliminary data, we propose that Rab40b is a dual-functioning Rab GTPase, given its co-regulation of vesicular MMP trafficking as well as Rap2 activity, and such co-regulation plays a key role in driving 3D cell migration and invasion. Results gained from this study will broaden our scientific understanding not only of Rab GTPase function but will also help uncover novel machinery governing cell migration.

P443/B453

Stochastic Activation and Bistability in a Rab GTPase Regulatory Network.

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Rab GTPases are the central regulators of intracellular traffic. Their function relies on their conformational change triggered by nucleotide exchange and hydrolysis. While this switch is well understood for an individual protein, how Rab GTPases collectively transition between different states is not known. Here, we combine in vitro reconstitution experiments with stochastic reaction-diffusion modeling to study the non-equilibrium properties of the minimal Rab5 activation network. We find that collective switching of Rab5 relies on a positive feedback and is triggered by intrinsic noise due to low amounts of Rab5[GDP] preexisting on the membrane. We show that this inactive population is not only the source of stochastic behavior of this biochemical circuit, but can act as a control parameter for Rab5 activation.

P444/B454

Determining the Effects of *Legionella Pneumophila* -Side Family Effector Proteins on Host Endocytic Recycling Systems.

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Legionella pneumophila is an opportunistic, intracellular pathogen responsible for Legionnaire's disease in humans. Upon infection, *L. pneumophila* forms the *Legionella* Containing Vacuole (LCV) to shield itself from host immune responses. From the LCV, *L. pneumophila* use a T4SS secretion system to secrete over 320 effector proteins into the host cell. It Is able to avoid phagocytosis by interfering with transport of "(ER)-derived vesicles" from ER to Golgi and using those vesicles to coat the outside LCV. These proteins

are essential for targeting normal host pathways to optimize the environment for *L. pneumophila* replication. One pathway that is disrupted after *L. pneumophila* infection is the endocytic recycling pathways. This system can use two distinct recycling pathways, a slow or fast route which use host cell Rab-GTPases to transport endosomes containing proteins and nutrients to the plasma membrane. Rab11a is an essential RabGTPase in the slow recycling pathway, and it works in concert with additional adapter proteins such as FIP1A, which aid in transferrin recycling. The Neunuebel lab has observed defects in transferrin recycling during *L. pneumophila* infection. We have identified an interaction between Rab11a and SidE family proteins. SdeA, a protein in this family, has been shown to ubiquitinate certain RabGTPases. We have found that SdeC interacts with Rab11a and localizes in close proximity to it. Further investigation revealed a stronger interaction between SdeC and Rab11a-FIP1A. This may be through ubiquitination of Rab11a-FIP1A by SdeC. In addition to Rab11a-SdeC interactions, were are interested in exploring the role of a family of Rab adapter proteins known as FIPs in recycling during infection and present data that FIP binding to Rab11 may be disrupted through SdeC. Overall we hope to understand the role of endocytic recycling pathways during intracellular bacterial infection.

P445/B455

Roles and Regulation of the Phosphatidylinositol 4-kinase Pik1 at the *Trans*-golgi Network.

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In eukaryotes, the *trans*-Golgi network (TGN) is a central hub for vesicle-mediated protein trafficking. At the *Saccharomyces cerevisiae* TGN, the small GTPases Arf1 (ARF1 homologue) and Ypt31 (Rab11 homologue) collaborate with an essential pool of phosphatidylinositol 4-phosphate (PI4P) to recruit vesicle coats, transport motors, and tethering proteins to nascent secretory vesicles. The conserved PI 4-kinase Pik1 (PI4KIIIβ homologue) generates PI4P at the TGN, and although its catalytic function is well-characterized, its interactions with other TGN regulatory factors are not. Using a combination of imaging and in *vitro* protein-protein interaction assays, we have found that Pik1 is recruited to the TGN by Arf1. Further, once there, Pik1 helps control recruitment of several downstream Ypt31 effectors, likely through its established binding interaction with Ypt31, to temporally regulate secretory vesicle formation. These results, together with ongoing structural work, paint a clearer picture of the mechanisms by which Pik1 works alongside Arf1 and Ypt31 to choreograph TGN function.

P446/B456

Mechanosensory Dendrite Termination and Refinement in *C. Elegans*.

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Exuberant axon and dendrite growth followed by stereotyped shortening is common during the development of neural circuits in most animals including humans. Despite the critical importance of this process, the underlying molecular mechanisms remain poorly understood. The posterior mechanosensory neuron (PLM) in *C. elegans* also follow this pattern of growth. PLM initially extends beyond its termination zone leading to overlap with its anterior counterpart, ALM. Its dendrite then retracts such that ALM and PLM divide the animal's body into anterior and posterior sensory domains ensuring proper response to localized touch. Importantly, dendrite termination and stereotyped shortening occurs just below the body surface during early larval development so PLM morphology can be measured in developing animals to identify important genes. Genes identified can then be monitored

at the sub-cellular level by tagging with fluorescent markers. In other words, PLM provides an excellent model for studying neurite termination and stereotyped shortening at sub-cellular resolution. My work first identified two genes conserved from yeast to humans: *sax-1* (an NDR kinase) and *sax-2* (a conserved scaffold protein). Null mutations in either gene lead to PLM overextension. Double mutant analysis indicates they function in the same pathway and a developmental time course comparing WT and *sax-2* mutants reveals a specific defect in the regulated shortening step of PLM dendrite development. More recently, we find that the *sax-1/sax-2* pathway may regulate polarized exocytosis. The *rab-10* GTPase and its GAP, *tbc-4*, also leads to PLM overextension and the *rab-10* null is **not** enhanced in the absence of *sax-1* or *sax-2*. Interestingly, endocytosis may also control PLM dendrite termination. *unc-11,* a worm ortholog of phosphatidylinositol binding clathrin assembly protein (PICALM) also leads to PLM overextension. PICALM functions in clathrin-coated endocytosis and VAMP recycling. Here, double mutant analysis indicates that *unc-11* functions in parallel to *sax-1* and *sax-2*. To better understand how these genes control PLM dendrite morphology, we have been fusing these genes to fluorescent markers using a CRISPR knock-in strategy. In preliminary work, we observe a small number of SAX-2::GFP puncta near the dendrite tip. We anticipate a fuller understanding of the genes that contribute to the establishment and maintenance of neural circuits will help guide rational design of therapies to aid people with spinal cord injuries or neurodegenerative disorders.

P447/B457

The an alysis of Rab32/38 Mediated Membrane Traffic in Macrophage and Osteoclast.

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Osteoclasts (OCLs) are multinucleated giant cells formed by repeated differentiation and fusion from the bone-marrow macrophages (BMMs). OCLs resorb bone by secreting vesicles and acids containing various proteases and enzymes such as tartrate-resistant acid phosphatase (TRAP) into the resorption pits formed on the bone surface. BMMs and OCLs are characterized of their dynamic inter cellular membrane traffic, however little is known about the underlying this mechanism. To understand the membrane how membrane traffic mechanism is involved in OCLs function, we focused on the Rab protein which is a regulator of membrane traffic. Firstly, we checked expression levels of all Rab proteins in BMMs and OCLs by using DNA microarray analysis. As a result, Rab38 expression levels significantly increased in OCLs. We focused on Rab38 and Rab32 which is paralogue of Rab38. To elucidate the physiological role of Rab32 and Rab38 in OCLs, we created Rab32 and Rab38 double knockout mice (Rab32/38 DKO mice) by using the Crispr/CAS9 system. Rab32/38 DKO mice have a phenotype with red eyes and ivory coat color. Then we analyzed the femur of 8-week-old wild type mice and Rab32 / 38 DKO mice by 3D micro CT analysis. As a result, Rab32 / 38 DKO mice had increased bone mass in femur was observed in the cancellous bone area. We confirmed whether deletion of Rab32 and Rab38 affects differentiation and bone resorption by using primary bone marrow-derived cultures. As a result, Rab32 and Rab38 deletion did not affect differentiation efficiency but decreased TRAP secretion during bone resorption. As a result of immunofluorescence staining in OCLs, Rab32 and Rab38 were co-localized on novel lysosomal like membranous organelles in BMMs and OCLs. Surprisingly this organelle dynamically

engulfed and degraded other organelles such as endosomes and mitochondria during differentiation process toward OCLs. Our findings indicate that Rab32 / 38 positive organelle is a novel lysosome-like organelle in OCLs. In addition, Rab32 / 38 positive organelles was involved in the biosynthesis of TRAP during bone resorption and have interesting properties that are also involved in endocytic pathway. OCLs function in bone resorption should be supported through these pathways.

P448/B458

Evidence That Rab18 and Class II Arfs Regulate Lipolysis and Adipose Triglyceride Lipase Association with Lipid Droplets in Hela Cells.

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Rab18 is a small GTPase that associates with lipid droplets, endoplasmic reticulum and other membranes of the secretory and endocytic pathways. While it likely has multiple functions on lipid droplets, both anabolic and catabolic, one proposed function is regulation of lipolysis. There is evidence for involvement of Rab18 in autophagy (Biochem. Biophys. Res. Commun 486(3):738-43; 2017) but whether it regulates lipid droplet catabolism via recruitment of cytoplasmic lipase is still unclear. We provide evidence that, in HeLa cells, Rab18 plays a role upstream of the cytosolic lipolytic enzyme adipose triglyceride lipase (ATGL). Furthermore, our evidence implicates elements of the Arf/GBF1 machinery in recruitment of ATGL by Rab18 and suggests a possible role for Class II Arfs. In HeLa cells loaded with 100 µM oleic acid for 24 hrs., we find that Arf4-GFP is specifically accumulated on the subset of lipid droplets associated with cerulean-Rab18 or mCherry-Rab18. This association is lost within 5 minutes of treatment with 5 µg/ml of the drug brefeldin A, which targets GBF1 and other Sec7-domain containing Arf exchange factors. ATGL-GFP is also recruited to lipid droplets, but is lost more slowly after treatment with 5 µg/ml brefeldin A. Loss of ATGL-GFP from lipid droplets is not significant after 5 min. Of BFA treatment, and significant but partial loss ($p < 0.001$) occurs after 1 hr treatment. Loss of detectable association of ATGL-GFP with lipid droplets was found 4 hrs after brefeldin a treatment. When the dominant negative GDP-locked cerulean-Rab18-S22N is overexpressed, GFP-ATGL and Arf4 are lost from the surface of lipid droplets similarly to BFA treatment. These results suggest a role for Rab18 in recruiting ATGL to lipid droplets.

Membrane Fission and Coat Proteins

P449/B459

Membrane Deformation Ability of the an kyrin Repeat and KH Domain-containing Protein 1 (ANKHD1) and Its Involvement in the Early Endosome Enlargement.

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Ankyrin-repeat domains (ARDs) are conserved in large numbers of proteins. ARDs are composed of ankyrin repeats (ANKs). The number of repeats varies among ARDs, and the sequence of ANKs often adopt curved structures reminiscent of the Bin-Amphiphysin-Rvs (BAR) domain, which is the dimeric membrane scaffold for membrane tubulation. BAR domains sometimes have amphipathic helices that enable membrane scission for vesicle formation. However, it is unclear whether ARD-containing proteins exhibit similar membrane deformation and scission properties. We screened the membrane deformation abilities of the highly expressed ARD proteins in vitro. We found that the ARD of ankyrin

repeat and KH domain-containing protein 1 (ANKHD1) dimerizes and efficiently deforms membranes into tubules and vesicles. ANKHD1 contains 25 ANKs that are divided into two groups: the first 15 ANKs and the latter 10 ANKs. The first 15 ANKs can form a dimer, and the latter 10 ANKs enable membrane tubulation and vesiculation. These 10 ANKs had an adjacent amphipathic helix and were predicted to have a curved structure with a positively charged surface, analogous to BAR domains. Mutations in positively charged amino acid residues and the deletion of the amphipathic helix abolished the membrane vesiculation ability. Interestingly, the dimeric 25 ANKs displayed significantly higher vesiculation ability than the 10 ANKs. Knockdown and localization of ANKHD1 suggested its involvement in the negative regulation of early endosome enlargement owing to its membrane deformation ability. These results indicate that ANKHD1 causes vesiculation of the early endosomal membranes in a manner similar to the BAR domain protein.

P450/B460

Septin Recruitment of Contractile Acto-myosin Cages to Vesicle Membranes Drives Regulated Exocytosis in Exocrine Glands of Live Mice.

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The constant remodeling of cellular membranes into various curved configurations is a requisite for myriad biological processes ranging from cytokinesis to intracellular trafficking. To elucidate the molecular machines and processes driving membrane remodeling under physiological conditions, we use the process of regulated exocytosis in the salivary gland of live mice as a model system. In this model, we recently showed that 1) F-actin and non-muscle myosin (NMII) form polyhedral-like lattices around large membrane-bound secretory granules (SGs) undergoing exocytosis, and 2) use contractility and polymerization-forces to push the SG membrane into the plasma membrane (integration), allowing content release into the extracellular space. This study raised new questions regarding how this unique acto-myosin network is both recruited and organized to perform its key function. To address this, we explored the role of septins, which we identified in a screen of purified SGs, as potential candidates, based on their known abilities to sense/generate micron-scale membrane curvature, interact with the actin cytoskeleton and recruit NMII. We found, strikingly, via immunofluorescence and super-resolution microscopy that septins 2, 6 and 7 (SEPT2, SEPT6 and SEPT7) are in fact present on the surface of fused SGs, where they each organize into cage-like lattices. Using transgenic mice expressing GFP-NMIIA, we show that NMIIA colocalizes with the SEPT2 and that pharmacological inhibition of SEPT2 results in a significant decrease in the presence of activated NMII and of myosin light chain kinase (MLCK) on actin-coated fused SGs. Conversely, disruption of F-actin assembly on the SG surface leads to an expansion in SG size without impairing NMII or septin recruitment. Finally, we find that the dynamics of integration and the levels of acto-myosin on the SG surface are both compromised in mice lacking SEPT7 expression in salivary gland secretory cells. Based on our data, we propose that the newly observed septin-lattices: 1) provide a molecular scaffold to recruit and curve acto-myosin filaments populating the surface of SGs, and 2) are needed for the activation of NMII, likely through MLCK-mediated phosphorylation, which ultimately drives SG exocytosis. Finally, the pattern of organization of both septins and NMII on the SG surface provide new structure-function insights into the molecular mechanisms driving membrane remodeling in *vivo*.

P451/B461

The Cryo-em Structure of the Snx-bar Mvp1 Tetramer.

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Sorting nexins (SNX) are a diverse family of lipid-binding proteins with pivotal roles in trafficking and signaling processes. The SNX-BAR subfamily has critical membrane-remodeling functions, particularly at the endosome. The SNX-BAR minimal module, consisting of a tight association of a PI3P-binding PX domain and a curvature generating BAR-domain, has been shown to be a dimer, formed by an extensive BAR-BAR interface. We use cryo-electron microscopy (cryo-EM) to determine the structure of the full-length SNX-BAR Mvp1 to a global resolution of 4.2 Å. Surprisingly, Mvp1 is an autoinhibited tetramer, consisting of a dimer of dimers, wherein both concave membrane-binding BAR dimer surfaces are sequestered into the interior of the tetramer and the lipid-binding sites of the PX domains are occluded. The N-terminal low-complexity region (~100 residues) of Mvp1 is disordered but plays a critical role in tetramerization, as its removal results in constitutive dimers that exhibit enhanced membrane association in *vivo* and increased membrane binding and tubulation activity in *vitro*. Tetramerization can also be abolished by point mutations within the PP_{II} loop of the Mvp1 PX domain. Membrane binding and remodeling by Mvp1 therefore requires unmasking of the PX and BAR domain lipid-interacting surfaces, which are occluded by tetramerization promoted by the N-terminus. The functional importance of the low-complexity N-terminus in regulation of tetramerization provides an explanation for why existing SNX-BAR crystal structures, generated from minimal PX-BAR constructs, are invariably dimeric. This work reveals a novel tetrameric configuration of a SNX-BAR protein that provides critical insight into SNX-BAR function and regulation.

P452/B462

The Timing and Localization of ESCRTS in Membrane Bending and Scission: Lessons from Studying Single Virions of HIV-1.

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Host cellular proteins that are members of the Endosomal Sorting Complexes Required for Transport (ESCRT) family are involved in many cellular processes that require bending of the membrane away from the cytosol. These include the formation of multivesicular bodies, cell scission at cytokinesis, repair of plasma and nuclear membrane, release of ectosomes and budding of retroviruses. Some hypotheses posit that the ESCRT-IIIs drive the bending of the membrane and some hypotheses posit that ESCRT-IIIs drive the membrane scission. We used the assembly of HIV-1, which occurs on the surface of cells as a model system for high resolution imaging of the timing and localization of the recruitment of ESCRT-III. We have quantified timing, the number and the localization of ESCRT-IIIs in HIV-1 while monitoring membrane bending and scission. The ESCRT-III arrive after the recruitment of the genome, and many minutes after the recruitment of the coat protein Gag and after the initiation of bending of the membrane This recruitment of ESCRT-IIIs is transient. They arrive roughly 60 seconds before scission, are at the site of virion assembly for 40 seconds, and can no longer be detected 20 seconds prior to scission. Using super-resolution microscopy, we find the ESCRT-IIIs are only recruited to the neck of the nascent virion with ~100 molecules recruited, enough to form one or two rings. The results indicate that ESCRT-IIIs play a critical role in the final steps of assembly, but they themselves are not responsible for initiating bending and they may not be providing the force for scission.

P453/B463

The Proppin Wipi1 Promotes Membrane Fission for Protein Exit from Endosomes.

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Endosomal and lysosomal compartments exchange material through tubulo-vesicular carriers, which recruit cargo by coat complexes such as retromer, retriever or commander. How these carriers separate from the endosomes is poorly understood. Here, we show that the PROPPIN family protein WIPI1 is required for protein exit from endosomes towards Golgi, lysosomes and plasma membrane. It promotes the formation and fission of endosomal membrane tubules through its two phosphoinositide binding sites. WIPI1 transport activity requires the lipid $PI(3,5)P_2$, one of the two phosphoinositide binding sites of WIPI1, and the formation of an amphipathic α-helix between these lipid binding sites. Inactivation of any one of these three features, which are all critical for the in vitro membrane fission activity of the yeast WIPI1 homolog Atg18, leads to a strong enlargement of endosomal compartments. The organelles then accumulate micrometer-long membrane tubules carrying endosomal markers and cargo proteins. WIPI1 may thus be implicated in membrane fission in multiple endosomal protein exit pathways, suggesting that membrane fission activity may be a general feature of PROPPIN family proteins.

P454/B464

Curvature Induction and Sensing of the F-BAR Protein Pacsin1 on Lipid Membranes Via Molecular Dynamics Simulations.

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F-Bin/Amphiphysin/Rvs (F-BAR) domain proteins play essential roles in biological processes that involve membrane remodelling, such as endocytosis and exocytosis. It has been shown that such proteins transform the lipid membrane into tubes. Notably, Pacsin1 from the Pacsin/Syndapin subfamily has the ability to transform the membrane into various morphologies: striated tubes, featureless wide and thin tubes, and pearling vesicles. The molecular mechanism of this interesting ability remains elusive. In this study, we performed all-atom (AA) and coarse-grained (CG) molecular dynamics simulations to investigate the curvature induction and sensing mechanisms of Pacsin1 on a membrane. From AA simulations, we show that Pacsin1 has internal structural flexibility. In CG simulations with parameters tuned from the AA simulations, spontaneous assembly of two Pacsin1 dimers through lateral interaction is observed. Based on the complex structure, we show that the regularly assembled Pacsin1 dimers bend a tensionless membrane. We also show that a single Pacsin1 dimer senses the membrane curvature, binding to a buckled membrane with a preferred curvature. These results provide molecular insights into polymorphic membrane remodelling.

P455/B465

Induced Nanoscale Curvature Localizes Endocytic Machinery and Rescues Clathrin Knockdown.

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Clathrin-mediated endocytosis (CME), the principal route of receptor internalization in eukaryotes, requires rapid, coordinated membrane restructuring on the scale of 50-100 nanometers to produce spherical vesicles from flat membrane. Here, we have used a combination of electron beam lithography (EBL) and UV-nanoimprint lithography (UV-NIL) to create a set of glass-like nanofabricated substrates, suitable for most optical microscopy techniques, with curved nanoscale features of diameters ranging from 50-1000 nanometers to mimic the stages of CME progression and beyond. We have imaged fluorescently tagged endocytic proteins from distinct stages of CME in SKMEL2 and MDA cells as they undergo endocytosis. We found that proteins from across stages of endocytosis are localized to sites of high curvature and that the lifetimes of early endocytic proteins AP2 and clathrin are shortened significantly as curvature increases. We found that the protein Epsin1, which binds directly to PIP_2 and generates curvature early during endocytosis, strongly localizes to sites of high curvature but does not turn over for the duration of our imaging, indicating it may be dispensable in the presence of induced curvature. The vesicular fission protein dynamin2 exhibits a two-fold increase in turnover rate as curvature increases, indicating that curvature-induced localization of endocytosis corresponds to an increased rate of CME. Together, these data suggest that generation of curvature may be rate-limiting in CME. To test whether exogenous curvature could rescue a defect in endocytosis, we knocked down clathrin using siRNA. Curvature-dependent localization of AP2 occurs in clathrin knockdown cells. We also found that curvature rescues the localization and turnover of dynamin2 in the background of both clathrin knockdown by siRNA and treatment by pitstop2, an inhibitor of CME that halts curvature progression. To test whether exogenous curvature rescues productive endocytosis from clathrin knockdown, we performed transferrin uptake assays and found that the presence of exogenous curvature partially rescues transferrin uptake in the context of 80% depletion of clathrin by siRNA. These data are consistent with clathrin acting as a Brownian ratchet, locking in the energetically costly membrane curvature as CME progresses, and that the presence of induced curvature may bypass this energetically costly step.

Polarity in Development

P456/B467

SGEF, a RhoG-specific GEF, Associates with the Scribble Polarity Complex and Regulates Cell-cell Junctions, Contractility and Lumen Formation in Epithelial Cells.

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The canonical Scribble polarity complex is implicated in regulation of epithelial junctions and apical polarity. However, little evidence is available regarding the molecular mechanisms that control its function. Here, we show that SGEF (SH3-containing Guanine nucleotide Exchange Factor), a RhoG-

specific GEF, interacts simultaneously with Scribble and Dlg1, and functions as a bridge that mediates the formation of a ternary complex. Our results show that SGEF is recruited to apical junctions in a Scribble-dependent fashion and functions in the regulation of actomyosin-based contractility and barrier function at tight junctions as well as E-cadherin-mediated formation of adherens junctions. Surprisingly, SGEF does not control the establishment of polarity. However, in 3D cysts, SGEF regulates the formation of a single open lumen. Interestingly, SGEF's nucleotide exchange activity regulates the formation and maintenance of adherens junctions, and in cysts, the number of lumens formed, whereas, SGEF's scaffolding activity is critical for regulation of actomyosin contractility and lumen opening. We propose that SGEF plays a key role in coordinating junctional assembly and actomyosin contractility by bringing together Scribble and Dlg1 and targeting RhoG activation to cell-cell junctions.

P457/B468

Cytokinetic Bridge Triggers *De Novo* Lumen Formation in *Vivo*.

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Multicellular rosettes are polarized, transient epithelial structures that serve as intermediates during the formation of diverse organs. We have identified a unique contributor to rosette formation in zebrafish Kupffer's Vesicle (KV) that requires cell division, specifically the final abscission stage of mitosis. KV utilizes a rosette as a prerequisite before forming a central lumen surrounded by ciliated epithelial cells. Our studies identify that KV-destined cells remain interconnected by cytokinetic bridges that position at the rosette's center, acting as a landmark for directed Rab11 vesicle motility to deliver an essential cargo for lumen formation, CFTR. We found that prematurely severing a bridge using laser ablation or inhibiting abscission using Rab11 optogenetic clustering results in disrupted lumen formation. We present a model in which mitotic cells within the KV rosette strategically place their cytokinetic bridges at the site of future lumen formation, where Rab11-associated vesicles transport CFTR to aid in lumen establishment.

P458/B469

Tracking Stereociliary Bundle Establishment in Utricular Explants of the Mouse Inner Ear.

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The orientation of the stereociliary bundle (hair bundle) on the apical surface of sensory hair cells (HCs) determines their directional selectivity. A hair bundle is comprised of a kinocilium, tethered by a staircase of microvilli known as stereocilia. Only when the hair bundle is deflected towards its kinocilium, the tallest rod in the bundle, results in opening of the mechano-transduction channels on the tips of the stereocilia and activates (depolarizes) the HC. Because of this property of the hair bundle, each sensory organ of the inner ear exhibits a defined hair bundle orientation pattern that is tailored for its function. Notably, in each of the two vestibular maculae, the utricle and saccule, hair bundles can be divided into two regions of opposite orientations by a line of polarity reversal (LPR). Previously, we have shown that the LPR is established by the restricted expression of a transcription factor Emx2 to one side of the LPR and thus changes the hair bundles within that region from the default position by 180 degrees. We investigated whether there is a fundamental difference in hair bundle establishment

between Emx2+ and Emx2- HCs by live imaging of embryonic utricular cultures, in which HCs are tdTomato-positive and centrioles are GFP-positive. Hair bundle establishment is thought to be initiated by the mother centriole docking at the apical HC surface to serve as the basis for the kinocilium. After kinocilium formation, it migrates to its destinated position in the periphery before the stereocilia staircase is built around it. We distinguished the two GFP-positive centrioles based on the fact the mother centriole is more apically located than the daughter centriole. Our result showed that the daughter centriole always migrated to the periphery from the center of the HC ahead of the mother centriole, suggesting that the daughter centriole may be guiding the mother centriole to its destinated location. Additionally, we did not find any obvious difference in the trajectory of centrioles to their destinated location between Emx2+ and Emx2- HCs, other than the fact that their final positions are on opposite side of the HC. These results suggest that the cellular machinery that guides directed centriole movements has already been established by downstream effector(s) of Emx2 prior to kinocilium migration to the periphery.

P459/B470

An Unexpected Role for the LGN Homolog AGS3 in Promoting Planar Divisions during Epidermal Morphogenesis.

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Asymmetric cell divisions, whereby progenitor cells divide to give rise to daughter cells that adopt different fates, are an important mechanism to promote cellular diversity. An evolutionarily conserved complex of polarity proteins directs asymmetric cell divisions, including the core scaffolding protein LGN (Gpsm2). The *Drosophila* LGN ortholog Pins plays a key role in both the positioning and orientation of the mitotic spindle in neuroblasts, ensuring the unequal inheritance of fate determinants that induces different daughter cell fates. In basal cell progenitors in developing stratified epithelia, we have shown that LGN localizes asymmetrically to the apical cortex and promotes perpendicular divisions. Epidermal LGN loss leads to elimination of perpendicular divisions, decreased differentiation and impaired barrier function, resulting in neonatal lethality. This highlights the critical importance of maintaining a proper balance between planar (symmetric) and perpendicular (asymmetric) divisions. While the complex of proteins that promotes perpendicular divisions has been well characterized, much less is known about what orchestrates planar divisions. One candidate is the LGN homolog AGS3 (Gpsm1), although whether AGS3 possesses intrinsic spindle orienting activity remains controversial. Surprisingly, our data show that AGS3 does play an important role in spindle orientation in the developing epidermis, and in fact appears to oppose LGN function. Loss of AGS3 increases perpendicular (asymmetric) divisions, while AGS3 overexpression increases the proportion of planar divisions. However, because AGS3 does not appear to be detectable or polarized at the cell cortex in mitotic basal cells—as previously shown in neuronal progenitors—we hypothesize that AGS3 may impact spindle orientation indirectly by competing with LGN for downstream binding partners. Supporting this, we observe that AGS3 knockdown increases LGN cortical intensity, while AGS3 overexpression disrupts LGN cortical localization. Finally, we have recently described a phenomenon termed “telophase reorientation” (https://doi.org/10.1101/668244), in which basal cells that enter anaphase at oblique angles correct to either planar or perpendicular during telophase. Using live imaging of epidermal explants, we show that LGN plays a maintenance role during telophase, promoting perpendicular reorientation. Interestingly,

AGS3 also appears to function during telophase reorientation, as a higher percentage of oblique divisions reorient to perpendicular during telophase upon AGS3 loss. Together, these data suggest that the two vertebrate Pins orthologs play opposing roles in spindle orientation in the developing epidermis.

P460/B471

The Role of Bloc-1 in Planar Cell Polarity.

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Planar cell polarity (PCP) regulates coordinated cellular polarity along an axis parallel to the plane of the tissue. PCP signaling is critical for developmental functions. Defects in PCP can result in mis-orientation of hair cell stereociliary bundles in the cochlear epithelium, deafness and neural tube closure. A key feature of PCP is the asymmetrical location of core PCP proteins, Vangl2 and Frizzled. Previously we showed that Adaptor Protein Complex -3 (AP-3) plays a role in the Vangl2 trafficking and PCP. However, the molecular mechanisms that regulate the sorting of PCP transmembrane proteins is not fully understood. Here, we investigate the role of biogenesis of lysosome-related organelles complex-1 (BLOC-1) in PCP and Vangl2 trafficking. To elucidate the underlying mechanisms of Vangl2 sorting, we evaluated the distribution of Vangl2 in cochlea from loss of function BLOC-1 mice, $Dtnbp1^{sdy/sdy}$ and $Pldn^{pa/pa}$. We observed a reduced membrane localization of Vangl2 in $Pldn^{pa/pa}$. We show notable PCP defects including mis-oriented hair cell sterociliary bundles and extra hair cell rows in these mice. These finding strongly suggest that BLOC-1 mediated endosomal pathways are essential for PCP sorting and membrane targeting of Vangl2.

P461/B472

Hematopoietic Stem Cells Polarise in Contact with Bone Marrow Stromal Cells by Engaging Cxcr4 Receptors.

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Hematopoietic Stem Cells (HSC) are located in the bone marrow, where they regulate the permanent production and renewal of all blood cell types. Their molecular and cellular micro-environment, called the niche, is a potent regulators of HSC proliferation and differentiation. However, the cellular mechanism of HSC interaction with the stromal cells of the niche has not been investigated in details. Here we used microfluidic compartments filled with hydrogels to reconstitute a bone-marrow on a chip as well as microwells to enforce a lasting contact between doublets formed by HSC and individual stromal cells. We found that HSC can become highly polarized in contact with niche stromal cells. They assembled a single, focused and thight anchorage point on the stromal cell from which they adopted the shape of a leaf of a wild cherry tree. As in the case of the immune synapses formed by lymphocytes, the centrosome was located in the proximity of the anchorage point. The entire microtubule network emmanated from the centrosome and appeared to keep the nucleus at a distance. HSC capacity to polarise was cell-type specific since it could be observed on endothelial cells and osteoblasts but not on fibroblasts. In addition, the frequency of polarised cells decreased with the degree of cell differentiation. Finally, we found that although ICAM, VCAM and CXCR4 were present in the anchorage point, only CXCR4 was intrinsically capable to trigger HSC polarisation. Altogether these results revealed a novel

interaction and polarisation mechanism of HSC which might be instrumental in the regulation of their fate.

P462/B473

Glutathione-s-transferase Pi (gstp) 1 and 2 Regulate Neurite Formation in *Vitro* and in *Vivo*.

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After neurons reach the cortical plate in the developing cerebral cortex, they start polarizing and extending neurites. Neurite initiation and elongation compose the process of neurite formation. This is an important step where neurons build mature structures and form networks with each other. Gstp family proteins are enzymes involved in reductive reactions, and therefore have an essential role in detoxification. Besides the enzymatic activity, Gstp proteins are also involved in cellular signaling. Studies on Gstp have mostly focused on the enzymatic functions, such as in cancer. However, little is known for the functions of Gstp proteins in brain development. In our study, we found that mouse Gstp proteins play important roles in the neurite formation. Knockdown of mouse Gstp1 and 2 in primary cortical neurons using shRNA caused a significant decrease in neurite number at DIV2 after re-plating. Knockdown of Gstp1 and *2 in vivo* by in *utero* electroporation at E15.5 enables us to label layer 2/3 cortical neurons and analyze neurite formation in *vivo*. We found Gstp1 and 2 knockdown caused further morphological defects in *vivo* including widening of apical dendrite at P3. Live imaging showed the polarity of knockdown neurons is disrupted at P0, but this disruption is not persistent at P3. Based on our observations, we conclude that the Gstp1 and 2 are involved in neurite formation, especially the neurite initiation stage. Some interaction partners for Gstp1 and 2 have been identified, for example JNK1 and Akt, and we are intrigued to discover the molecular targets and signaling pathway responsible for the neurite formation defects seen in Gstp1 and 2 knockdown neurons.

P463/B474

***Lgl1* Is Essential for Collective Epithelial Migration by Promoting Integrin Singaling during Mammary Gland Branching.**

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Tissue polarity is a salient feature of epithelial organs and its loss is a hallmark trait of cancer, and *Lgl1* is an important protein in the epithelium to maintain tissue polarity. The use of mammary gland epithelial tissue to study the function of *Lgl1* can further reveal the regulatory mechanism and function of epithelial tissue polarity. Here, we show that *Lgl1* expression is reduced in breast cancer and its extent of reduction correlates with the severity of cancer grades. Consistent with it being a candidate tumor suppressor, *Lgl1* null cells showed higher self-renewal ability in mammosphere- and acinus-forming assays. Surprisingly, conditional removal of *Lgl1* function or transplantation resulted in stunted epithelial branching. Although *Lgl1* null cells could undergo FGF2-mediated ductal elongation, they failed in collective epithelial invasion, an essential step in vertebrate branching. To determine the molecular mechanism of failure in invasion due to loss of *Lgl1*, we discovered that ITGB1 had new interactions with Lgl1 in mice, and *Lgl1* regulates the invasion process by activating the integrin signaling pathway. Therefore, the function of tissue polarity protein Lgl1 may relate to the cellular microenvironment.

Together, our data highlights the importance of tissue polarity and actin dynamics in epithelial organ development and suggests that its loss is an essential step in solid tumor formation.

P464/B475

Linking Symmetry Breaking to Asymmetric Division in the Stomatal Lineage.

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Symmetry breaking is essential to coordinate cellular behaviors to pattern tissues in all multicellular organisms. However, our understanding of asymmetry-generating mechanisms in plants remains limited. Stomata, pores in the leaf epidermis that mediate gas exchange, are patterned by asymmetric divisions of precursor cells. In *Arabidopsis*, symmetry breaking in these cells is initiated by the polarization of BASL and BRX, plant-specific proteins that localize in a cortical crescent. This polarity domain is essential for proper division orientation and differential daughter cell fates. However, the mechanisms that link their polarization to asymmetric division remain unknown. By combining quantitative, long-term time-lapse imaging with pharmacological and genetic perturbations, we have uncovered two novel, cytoskeleton-driven pathways that link symmetry breaking to asymmetric division in plants. In one pathway, precursor asymmetry required mutual inhibition between the polarity crescent and membrane-associated microtubules. We found that anticlinal microtubules, while not required for initial polarization, restrict lateral spread of the polarity domain, conferring robustness. In turn, polarity proteins locally altered microtubule organization by suppressing microtubule stability at the membrane. Local microtubule depletion resulted in microtubule network anisotropy that is propagated through the cell cycle to position the division plane. In a parallel pathway, symmetry breaking at the membrane drives directional nuclear migration before and after division via a two-step "cytoskeletal hand-off" mechanism. We found that BASL and BRX repel the nucleus in a microtubule-dependent manner before division but attract it in an actin-dependent manner following division. This coordinated nuclear movement potentially primes consecutive asymmetric divisions to ensure that daughter cells are properly distributed throughout the leaf epidermis. Taken together, we have uncovered an evolutionarily divergent polarity system that provides a framework to understand how symmetry breaking and asymmetric cell division are coordinated in plants.

Synaptic Cell Biology

P465/B477

The G Protein-coupled Receptor Fshr-1 Regulates Neuromuscular Synapse Structure in *C. Elegans*.

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G protein-coupled receptors (GPCRs) are a class of transmembrane proteins involved in diverse processes across eukaryotes and are common drug targets. In the nervous system, GPCRs are receptors for numerous signaling molecules and can impact neurotransmitter release. Follicle Stimulating Hormone Receptor (FSHR) is a conserved GPCR expressed in *Caenorhabditis elegans* neurons that modulates neuromuscular signaling. In *C. elegans,* a balance of excitatory signals released from cholinergic motor neurons and inhibitory signals released by GABAergic neurons controls muscle contraction. Inhibition of *fshr-1* expression causes reduced muscle contraction and synaptic vesicle accumulation at cholinergic, and to a lesser extent, at GABAergic motor neuron presynapses, suggesting

decreased neurotransmitter release. Yet, the mechanisms by which FSHR-1 signaling impacts synaptic vesicle function are unknown. Active zone (AZ) proteins are located at presynaptic membranes and regulate synapse structure, synaptic vesicle clustering, and neurotransmitter release. As the mammalian FSHR signaling target protein kinase a can regulate the AZ protein RIM, we hypothesized FSHR-1 might regulate synaptic vesicle localization and neuromuscular signaling in *C. elegans* via effects on UNC-10/RIM or other AZ proteins. To test this, we imaged neuromuscular synapses within the dorsal nerve cords of wild type and *fshr-1* loss-of-function mutant worms expressing fluorescently tagged AZ proteins in specific motor neuron classes and quantified relative synaptic AZ protein distribution. Compared to wild type worms, *fshr-1* mutants had increased synaptic UNC-10::GFP intensity (p = 0.02) and elevated axonal fluorescence (p = 0.02) in cholinergic motor neurons, suggesting *fshr-1* is required to prevent aberrant UNC-10 accumulation at these synapses. No such differences were seen for UNC-10::Cherry in GABAergic motor neurons. To assess if the defects were specific to UNC-10, we measured the localization/abundance of other active zone proteins. *fshr-1* mutants showed slightly elevated synaptic SYD-2/Liprinα::GFP intensity (p = 0.05) and axonal fluorescence (p= 0.04) in cholinergic neurons, following a similar but less robust trend than seen with UNC-10::GFP. *fshr-1* mutants expressing CLA-1/Clarinet::GFP showed no significant differences from wild type worms in cholinergic neurons. Together these data suggest that FSHR-1 may regulate neuronal communication and synaptic vesicle release through effects on UNC-10 and SYD-2. Future studies will examine the mechanisms by which FSHR-1 controls synaptic UNC-10 and SYD-2 levels. Given the conservation of *C. elegans* and human genes/nervous system organization, elucidating FSHR-1 biology may aid knowledge of human neurological disorders.

P466/B478

Exploring the Fshr-1 Gpcr Signaling Pathway in Controlling *C. Elegans* Neuromuscular Signaling.

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A tightly regulated balance of excitatory and inhibitory (E:I) neuronal signals is essential for nervous system function; E:I imbalances occur in many neurological diseases. G-protein coupled receptors (GPCRs), which activate multi-protein pathways in diverse cell types in response to diverse signals, are important regulators of this balance. Given the high number of GPCRs and downstream components, the function and members of many GPCR pathways in specific cell types are unknown. This is particularly true in the nervous system, where GPCRs can serve as receptors for molecules including neurotransmitters and neuropeptides. FSHR is a mammalian GPCR that regulates reproductive physiology via its expression in the gonad; however, FSHR is also expressed in nervous tissue, where its function is unknown. The *C. elegans* homolog FSHR-1 is also expressed in neurons where it controls E:I balance at the neuromuscular junction (NMJ) in these roundworms. At the *C. elegans* NMJ, excitatory acetylcholine (Ach) signaling and inhibitory γ-aminobutyric acid (GABA) signaling control muscle contraction. The specific targets of FSHR-1 at the NMJ and its downstream pathway components in this context have not yet been elucidated. In the *C. elegans* germline, FSHR-1 acts upstream of the Gαs protein GSA-1 and the adenylyl cyclase ACY-1, and a GSA-1—ACY-1—PKA pathway controls synaptic vesicle release in motor neurons. We investigated whether FSHR-1 activates this pathway to affect muscle contraction. Our preliminary data suggest FSHR-1 can act in multiple cell types, including Ach and GABA motor neurons, to control muscle excitation via effects on synaptic vesicle release; however, experiments testing the potential FSHR-1 pathway indicated that, while FSHR-1 acts upstream of GSA-1

at the NMJ, ACY-1 and PKA may not function downstream of FSHR-1 in one or more of these cell types. Three family members of ACY-1 – ACY-2, -3 and -4 – are structurally similar to ACY-1, and expression of genes encoding several of these proteins has been reported in neurons. Thus, we hypothesize that ACY-2, -3, and/or -4 may act downstream of FSHR-1 and upstream of PKA in one or more NMJ cell types. Using RNA interference and aldicarb paralysis assays, we found a requirement for *acy-3* in GABA neurons, as well as confirmed a requirement for genes encoding *unc-2* (CACNA1B), *unc-10* (RIM1), and *egl-36* (KCNC1, 2 and 4) for normal muscle contraction at the *C. elegans* NMJ. Current experiments are assessing these genes and PKA for roles downstream of FSHR-1 and will examine the potential activation of this pathway by neuropeptide- and FSH-related genes. Given the similarity between worm and human genes and nervous systems, this research provides insight into the molecular mechanisms by which GPCRs like FSHR-1 control neuronal signaling.

P467/B479

Neto-mediated Intracellular Interactions Sculpt Postsynaptic Composition at the *Drosophila* Neuromuscular Junction.

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Recruitment of neurotransmitter receptors and organization of postsynaptic densities (PSDs) are crucial for the formation of neural circuits and for the long-term plasticity underlying learning and memory. *Drosophila* neuromuscular junction (NMJ), a glutamatergic synapse similar to our central synapse, is a powerful genetic system to study synapse assembly and function. We have previously found that trafficking and stabilization of the ionotropic glutamate receptor (iGluRs) at the *Drosophila* NMJ requires Neto, a highly conserved auxiliary protein. Neto binds to iGluRs and modulates their function, but also engages in intracellular and extracellular interactions that shape PSD composition and enable iGluRs stabilization at synaptic sites. *Drosophila neto* codes for two isoforms, Neto-alpha and Neto-beta, which have distinct cytoplasmic domains generated by alternative splicing. Neto-beta, the predominant isoform at the larval NMJ, is key to the recruitment of iGluRs and PSD components such as p21-activated kinase (PAK), a PSD component known to selectively stabilize type a glutamate receptors. Here we propose that the cytoplasmic domain of Neto-beta provides a signaling platform required for PSD assembly and function. To test this hypothesis, here we have used CRISPR/Cas-9 and generated a series of *neto-beta* alleles with progressively truncated intracellular domains, or with small internal deletions. The 351-residue intracellular part of Neto-beta contains multiple putative phosphorylation sites and docking motifs, including an SH3 binding motif and a putative CaMKII phosphorylation site. Loss of the entire Neto-beta intracellular domain or truncations which remove the SH3 binding and the CaMKII putative phosphorylation site abolished the synaptic recruitment of dPix, a Rho-type guanine nucleotide exchange factor required for synaptic recruitment of PAK, and thus stabilization of type a glutamate receptors at the PSD. Interestingly, dPix recruitment was also disrupted by a small, 11 residue deletion which removes the putative CaMKII phosphorylation site. We will discuss the unexpected phenotype of this small deletion and the role of this Neto-beta motif on PSD assembly and NMJ functionality. Our study demonstrates that Neto functions as a critical PSD scaffold and a hub for cellular signaling at glutamatergic synapses.

P468/B480

Neto-α Controls Synapse Organization and Homeostasis at The*Drosophila*NMJ.

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Formation of functional synapses during development, and their fine-tuning during plasticity relies on ion channels and their accessory proteins. Auxiliary proteins are transmembrane proteins which associate with channel complexes and mediate their properties and distribution. Neto proteins are highly conserved auxiliary subunits which modulate the kainate-type glutamate receptors (KARs). In mammals, Neto/KAR complexes are critical for synapse plasticity but the underlining mechanism are extremely difficult to study due to the low abundance of these channels and the small currents they elicit. In contrast, Drosophila utilizes Neto and KARs at the NMJ, a synapse essential for viability. We demonstrated that Neto is required for synapse assembly and function at fly NMJ: in the absence of Neto, the NMJ iGluRs fail to cluster at the nascent synapses and the mutant animals die as paralyzed embryos. Here we show that Neto evolved functionally distinct isoform to modulate synapse development and homeostasis. *Drosophila neto* encodes two isoforms, α and -β, which share the extracellular and transmembrane parts but have distinct intracellular domain. Loss of Neto-β, the predominant isoform at the fly NMJ, drastically reduces the levels of post-synaptic receptors; however, these mutants have normal evoked potentials due to a robust compensatory increase of neurotransmitter release. Neto-α represents less than 10% of the total Neto at the NMJ and *neto-α^{null}* mutants have reduced evoked potentials and lack the ability to compensate for chronic or acute reduction of iGluRs activity. Interestingly, these mutants retain the ability to attenuate the excitatory effects of excess glutamate release. Our studies indicate that Neto-α functions in both pre- and post-synaptic compartments. In muscle, Neto-α limits the size of the postsynaptic receptors field. In motor neurons, Neto-α controls basal neurotransmission in a KAR-dependent manner. Furthermore, Neto-α is both required and sufficient for the presynaptic increase in neurotransmitter release in response to reduced postsynaptic sensitivity. This KAR-independent function of Neto-α is involved in activity-induced cytomatrix remodeling. Neuronal expression of Neto-β cannot rescue the defects of *neto-α^{null}* because Neto-β cannot traffic to the synaptic terminals. In contrast, a Neto variant with no intracellular domains (dCTD) can reach the presynaptic terminal, can rescue the basal neurotransmission defects of *neto-α^{null}*, but cannot restore their homeostatic response. Our studies indicate that the intracellular part of of Neto-α functions as a bona fide effector of synapse homeostasis. We propose that *Drosophila* ensured NMJ functionality by acquiring two Neto isoforms with different expression patterns and activities.

P469/B481

Ayahuasca Increases the Expression of Glur1 and Glur2/3 Ampa Receptors in the Hippocampus and Induces Opposite Changes in the Prefrontal Cortex of Wistar Rats.

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The consumption of Ayahuasca beverage is usual in several Brazilian syncretic religions that have expanded to Europe and North America. It is made from an Amazonian psychoactive plant containing the serotonin agonist N,N-dimethyltryptamine and the monoamine oxidase-inhibiting alkaloids (harmine, harmaline and tetrahydroharmine), resulting in enhanced serotonergic activation. Ayahuasca has also been reported to affect glutamatergic processes in brain areas including prefrontal cortex (PFC) and hippocampus, which play important roles in the neuropsychiatric disorders. This work aimed at investigating whether the ingestion of Ayahuasca might induce alterations in the expression of glutamate AMPA receptors (GluR1 and GluR2/3) in the hippocampus and dorsolateral PFC of rats. Six groups of male Wistar rats (230-250g, n=5-8/each) received 0.2 or 0.4ml/g of Ayauhasca, only once (acute), 3 times/day/3 days (subchronic) or once/day/15 days (chronic). Six control groups received water at the same conditions. After the last ingestion the animals were anaesthetized, perfused and their brains sectioned (40μm) for immunohistochemistry. The number of immunopositive cells (IC) was quantified, bilaterally, in the hilus of dentate gyrus (HDG), CA3, CA1 and in the superficial and deep layers of the PFC. Comparisons used ANOVA followed by Bonferroni, DMS and Duncan tests ($p \leq 0.05$). The acute ingestion of Ayahuasca (0.2ml/g) induced a decrease in GluR1 IC in CA1 (18%), while 0.4ml/g (acute or chronic) induced an increase in the HDG (30% and 29%) and CA1 (10% and 23%), respectively. Subchronic treatment showed no difference. For GluR2/3 IC, only chronic ingestion of either 0.2ml/g or 0.4ml/g of Ayahuasca induced an increase in CA3 (25%) and CA1 (20%). Acute, subchronic or chronic ingestion of 0.2 or 0.4ml/g of Ayahuasca induced an increase in GluR1 IC in both superficial and deep layers of the PFC (6-21%, $p<0.01$). However, for GluR2/3 IC, acute and subchronic treatments induced a decrease (7-15%, $p<0.01$) in both layers, while only the chronic ingestion of 0.2ml/g induced an increase (17-23%, $p<0.01$). No difference was found after chronic ingestion of 0.4ml/g. When the Ayahuasca groups were compared, the ingestion of 0.4ml/g always induced lower expression in both GluR1 and GluR2/3 than 0.2ml/g (7-18%, $p<0.001$). Acute or chronic ingestion of Ayahuasca may trigger distinct mechanisms in the hippocampus and PFC involving the modulation of glutamate through the activation of serotonin. Considering the involvement of both areas in several neurodegenerative and psychiatric disorders, glutamate might be a potential therapeutic target for the treatment of disorders where the glutamatergic dysfunction is associated with the serotonergic system activation. Support: FAPESP, FACISB

P470/B482

Selective Disruption of Bmp Signaling by a Smad Mutation Adjacent to the Highly Conserved H2 Helix.

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Bone morphogenetic protein (BMPs) modulate a wide variety of cellular processes via canonical and non-canonical signaling pathways. BMP signaling is initiated by ligands binding to type I and type II BMP receptors, and transduced by BMP effectors, Smads (Mad in *Drosophila*), which upon phosphorylation translocate in the nucleus and modulate gene expression. In flies, a canonical BMP pathway regulates neuromuscular junction (NMJ) growth and function. We have recently described a genetically distinct BMP signaling modality characterized by accumulation of phosphorylated Mad (pMad) at synaptic sites. This local BMP signaling functions as a sensor of synapse activity: it monitors active type-A postsynaptic glutamate receptors and acts locally to promote the stabilization of these receptors at synaptic terminals. To learn more about molecular features of Mad and understand how pMad could remain associated with its own (BMP receptor) kinase, we searched through available *Mad* alleles for mutants which differentially disrupt the two BMP signaling pathways. Within this comprehensive collection, we

found that strong *Mad* alleles generally disrupt both synaptic and nuclear pMad accumulation, whereas moderate *Mad* alleles have a wider range of phenotypes and selectively impact different BMP signaling modalities. In particular, Mad^{8}, which contains a single point mutation, S359L, showed drastically reduced synaptic pMad levels but mildly diminished nuclear pMad signals. The postsynaptic composition and electrophysiological properties of Mad^{8} NMJs were likewise altered. Using a cell-based assay, we found that pMad, but not pMad8, readily associates with activated BMP type I receptors at the cell membrane. Furthermore, structural modeling suggests that a highly conserved motif adjacent to S359, the H2 helix, is a critical determinant for the Smad-Type I receptor interaction. Several genetic variants identified in human patients map to H2, underscoring the relevance of this motif for normal development and function. This study demonstrates that the H2 helix is a class specific molecular determinant for local BMP signaling and suggests that mutations in this region disrupt specialized tight junctions throughout the animal kingdom.

P471/B483

Cytoskeletal Remodeling Regulates Human Fetal Synaptogenesis.

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Excitatory synapse formation occurs during mid-fetal gestation. However, due to our inability to image fetal synaptogenesis, the pre-natal period remains understudied. The recent development of human brain spheroids provides access to this critical period. Using human neurons and brain spheroids, we address how altered actin regulation impacts the formation of excitatory synapses during fetal brain development. We demonstrate that inhibition of RhoA kinase (ROCK) signaling promotes neurite formation and elongation. In addition to increasing neural complexity, ROCK inhibition increases spine precursor length. These increases correspond with increased excitatory synapse formation in human brain spheroids. Rac-driven actin polymerization drives this increase in excitatory synaptogenesis, and supports spontaneous action potential formation. Using STORM super-resolution microscopy, we localize key upstream RhoGTPase regulators (GEFs, GAPs) to nascent excitatory synapses, providing evidence for differential actin regulation at pre- and pos-synaptic compartments of emerging synapses. These results demonstrate that coordinated RhoGTPase activities underlie fetal excitatory synaptogenesis, and identify critical regulators of early synaptogenic events.

P472/B484

Presynapse Active Zones Assemble through Phase Separation of Scaffolding Molecules.

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The presynaptic active zone is the site of release of vesicles containing neurotransmitters. Components of the active zone are responsible for tethering and priming synaptic vesicles, recruiting and clustering ion channels to respond to action potentials, and structurally stabilizing the synapse through transmembrane connections to the postsynaptic cell. Decades of study have revealed the protein components of active zones, as well as a number of protein-protein interactions between them. Yet how these components assemble into functional active zones down a distant axon is not clear. Here, we show that two widely conserved active zone components in C. elegans, SYD-2/Liprin-α and ELKS-1, undergo a liquid-liquid phase separation to assemble the active zone. To probe active zone protein behavior in *vivo*, we measured protein mobility in newly formed *C. elegans* synapses. We found that

young active zones are dynamic and recover quickly after photobleaching, consistent with a possible phase separation. In contrast, established active zones are highly static with nearly zero cytoplasmic exchange. We find that SYD-2/Liprin-α and ELKS-1, two core active zone scaffolding proteins, are capable of liquid-liquid phase separation in *vitro* and rely upon multiple unstructured motifs for this activity. By selectively deleting short regions in these motifs, we specifically blocked phase separation activity without affecting other known functions of the proteins. When introduced endogenously in *vivo*, these mutations inhibit the early dynamics of developing synapses, supporting a phase separation during normal active zone assembly. We find that SYD-2/Liprin-α phase separation-blocking mutants localize normally to synapses; however, by imaging synapses of the Hermaphrodite Specific Neuron (HSN), we find additional active zone components fail to localize properly and synaptic vesicles fail to appropriately cluster. These defects in HSN active zone assembly lead to defects in its functional output of egg-laying, underscoring the importance of a phase separation in building the presynaptic active zone. Together these data show presynaptic active zones are assembled through a phase separation of core scaffold molecules. This mechanism may aid in tightly concentrating scaffolding proteins at nascent synapses, forming a potent sink to recruit binding partners and downstream active zone components.

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Mitochondrial Calcium Store and Downstream NAD(H) Redox Homeostasis Modulates Hair-cell Presynapse Size during Synapse Formation.

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Mechanosensitive hair cells of the inner ear and lateral line relay sensory information to the brain for vital behaviors such as hearing, balance and predator avoidance. Hair cells are epithelial cells that can transmit sensory information with specialized synapses, which have membraneless, proteinaceous “ribbons” anchored at the presynaptic active zone. Recent studies have shown that ribbon size can directly regulate the properties of this synapse. The main component of the ribbon is the protein Ribeye, which contains a NAD(H) binding domain shown to alter Ribeye self-aggregation in vitro. Our study aims to understand if cellular NAD(H) redox can impact Ribeye self-aggregation and ribbon formation determination in vivo. To study hair cell presynapse formation in vivo, we use transgenic larval zebrafish expressing fluorescent calcium biosensors and NAD(H) redox indicators in hair cells to visualize calcium dynamics and the NAD^+/NADH ratio. Our work also uses pharmacology to disrupt hair cell calcium and NAD(H) redox homeostasis. To quantify ribbon morphology we image immunofluorescence staining of Ribeye using Airyscan confocal microscopy. In developing hair cells, we found that application of exogenous NAD^+ or NADH increased or decreased the NAD^+/NADH ratio in hair cells. Furthermore, NAD^+ or NADH increased and decreased ribbon formation respectively. Additionally we found that in developing hair cells, spontaneous presynaptic and mitochondrial calcium uptake control the NAD^+/NADH ratio; these calcium signals also strongly affect ribbon formation. These results suggest that spontaneous presynaptic and mitochondrial calcium uptake modulate ribbon formation through depressing NAD^+/NADH ratio. This change in NAD^+/NADH ratio indicates that in hair cells, spontaneous activity may alter cellular metabolism to control development. Overall, these results propose a physiological mechanism that allows calcium influx and NAD(H) redox to regulate presynapse morphology.

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Glyoxal, a Potent Substitute for Paraformaldehyde/glutaraldehyde Fixation in Modern Electron Microscopy.

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Chemical fixation is one of the most critical steps to retain cellular structure and histochemical integrity. Recent advances with glyoxal as a fast fixative resulted in better structural preservation and better antigenicity in light microscopy and super-resolution microscopy. Those studies reported an increase in labeling intensity in immunolabelling of up to 100%. The penetration depth of antibodies was also substantially improved. Here, we present the very first data of modern glyoxal fixation applied to electron microscopy. We used primary hippocampal cell culture as our model and compared glyoxal fixation with established fixation techniques for electron microscopy. After classical fixation with glyoxal, we found similar improvements in labeling intensity with antibodies as reported for super-resolution. We used immunogold localization of synaptic vesicle proteins to quantify labeling intensity (gold particle per synaptic vesicle). When we used modern glyoxal fixation in which acids have been removed from commercial glyoxal, the fine structure was excellently preserved and was comparable if not better compared with established electron microscopic procedures.

P475/B487

Molecular Tuning of the Axonal Mitochondrial Ca^{2+} Uniporter Ensures Metabolic Flexibility of Neurotransmission.

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The brain is a vulnerable metabolic organ and must adapt to different fuel conditions to sustain cognitive function. Activity drives local ATP production in nerve terminals through activation of glycolysis and oxidative phosphorylation. Nerve terminals are a locus of metabolic vulnerability but how they regulate ATP synthesis as fuel conditions vary is unknown. We previously demonstrated that when glucose is present, glycolysis is stimulated in firing nerve terminals through recruitment of the glucose transporter GLUT4 to presynaptic membrane. Here, we show that synapses can switch from glycolytic to oxidative metabolism, but to do so, they rely on activity-driven presynaptic mitochondrial Ca^{2+} uptake to accelerate ATP production. The mitochondrial Ca^{2+} uniporter (MCU) which mediates Ca^{2+} uptake is reported to have a low affinity for Ca^{2+} in non-neuronal cells and relies on Ca^{2+} release from ER to open. We demonstrate that unlike non-neuronal cells, presynaptic mitochondria do not rely on the ER as a source of Ca^{2+} during uptake. In fact, axonal mitochondria readily take up Ca^{2+} in response to small changes in cytosolic Ca^{2+}, while in non-neuronal cells, mitochondrial Ca^{2+} uptake requires elevated extramitochondrial Ca^{2+}. We identified the brain-specific regulator of the MCU, MICU3, as a critical driver of this tuning of Ca^{2+} sensitivity. Knockdown of MICU3 lowers the Ca^{2+} sensitivity of axonal mitochondria similar to non-neuronal mitochondria, prevents acceleration of local ATP synthesis, and impairs presynaptic function under oxidative conditions. Conversely, overexpression of MICU3 in non-neuronal cells significantly lowers the threshold for mitochondrial Ca^{2+} uptake, suggesting that MICU3 is both sufficient and necessary for regulating the Ca^{2+} sensitivity of presynaptic mitochondrial Ca^{2+} uptake. Thus, presynaptic mitochondria rely on MICU3-driven mitochondrial Ca^{2+} uptake during activity to accelerate ATP production and achieve metabolic flexibility. The mechanisms underlying the metabolic

plasticity of mammalian brain serve to ensure that cognitive performance is faithfully maintained in the face of changing nutrient availability.

P476/B488

Demonstrating Synaptic Vesicle Recycling *Via* Clathrin Coated Vesicles in Mammalian Neuromuscular Junctions and Central Synapses.

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The concept that synaptic vesicles are the neuronal organelles that mediate quantal secretion of neurotransmitter has been accepted for decades now, and has been "proven" by a wide range of electron microscopical techniques. However, still under investigation are various critical aspects of how exactly synaptic vesicles are formed in the first place, and what happens to them after they discharge their transmitter quanta. Still, it is not possible to "stop-frame" the activity of an intact in *situ* neuronal net, and then to recognize and characterize all the various stages in synaptic vesicle formation, discharge, and recycling that must occur in healthy synapses. To finally accomplish this goal, we here return to the very start of this field of research, when we first introduced the concept of synaptic vesicle recycling by showing that the trivalent cation lanthanum (La+++) tremendously stimulated spontaneous transmitter release at the frog neuro-muscular junction (NMJ), and thereby exaggerated all the vesicle-transformations that are associated with neurotransmitter release. To accomplish this again here, we introduce a new mammalian neuromuscular preparation that is ideal for studying this process in the electron microscope - the intrinsic muscle of the mouse's ear - and we use this new preparation to show that La+++ mobilizes vesicle recycling in the 'cleanest' possible way, without causing any collateral damage to the synapse. We then carry this technique into CNS synapses, using tissue-cultures of mouse hippocampal neurons, to show that La+++ stimulates synaptic vesicle recycling in central synapses just as it does at the NMJ, and works in the same way, and with the same consequences - namely, *via* the turnover of synaptic vesicles mediated by clathrin-coated vesicle formation from the presynaptic membrane, after vesicle discharge. These new observations further establish our original proposal for how synaptic vesicles recycle as the proper modus operandi for future work aimed toward deeper understanding of the molecular basis of synaptic transmission.

P477/B489

Ultrafast Synaptic Vesicle Dynamics during Neurotransmitter Release.

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Synaptic vesicles fuse with the plasma membrane to release neurotransmitter following an action potential, after which new vesicles must refill vacated release sites. How many vesicles can fuse at a single active zone, where they fuse within the active zone, and how quickly they are replaced with new vesicles is not well-established. To capture synaptic vesicle exocytosis at cultured mouse hippocampal synapses, we induced single action potentials by electrical field stimulation then subjected neurons to high-pressure freezing to examine their morphology by electron microscopy. During synchronous release, multiple vesicles can fuse at a single active zone; this multivesicular release is augmented by increasing the extracellular calcium concentration. Synchronous fusions are distributed throughout the active zone, whereas asynchronous fusions are biased toward the center of the active zone. Immediately after stimulation a large fraction of plasma membrane-attached (docked) vesicles are lost,

both due to fusion and undocking. Between8 and 14 ms, new vesicles are recruited to the plasma membrane and fully replenish the docked pool, but docking of these vesicles is transient and they either undock or fuse within 100 ms. These results demonstrate that recruitment of synaptic vesicles to release sites is rapid and reversible.

P478/B490

A Specific Splice Isoform of Dynamin-1 Mediates Ultrafast Endocytosis.

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Membrane remodeling is a basic property of eukaryotic cells which generates a variety of structures, such as vesicles from the plasma membrane or daughter organelles from preexisting organelles. Following membrane invagination, GTPase dynamin family member proteins mediate fission of internalized structures. The timescale of dynamin-mediated membrane scission is thought to be at least 5-10 seconds. However, recently identified ultrafast endocytosis occurs in less than 100 ms during synaptic vesicle recycling in hippocampal neurons. Thus, the molecular mechanism underlying the rapid cleaving of this endocytic pit from the plasma membrane is not known. To reveal molecular underpinnings of this process, we coupled genetic depletion with flash-and-freeze microscopy, which visualizes membrane dynamics of synapses in electron micrographs with millisecond temporal resolution. Our data show that a specific splice variant of GTPase Dynamin-1 is required at the plasma membrane to retrieve endocytic vesicles within 100 ms after synaptic vesicle fusion. Dynamin 1 is recruited to the endocytic pit by Syndapin 1, an F-BAR protein which functions during the early stage of membrane invagination. Further, the kinetics of constriction require the interaction of Dynamin 1 and Syndapin 1. During Dynamin 1-dependent rapid membrane scission, Endophilin is also needed to stabilize and constrict steep negative curvature at the base of the endocytic pit. Thus, despite its intrinsic speed, it is the coordinated action of dynamin and its interacting partners which regulate rapid membrane cleaving during ultrafast endocytosis.

P479/B491

Maturation and Clearance of Autophagosomes in Neurons Depends on a Specific Cysteine Protease Isoform, Atg-4.2.

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In neurons, defects in autophagosome clearance have been associated with neurodegenerative disease. Yet, the mechanisms that coordinate trafficking and clearance of synaptic autophagosomes are poorly understood. Here, we use genetic screens and in *vivo* imaging in single neurons of *C. elegans* to identify mechanisms necessary for clearance of synaptic autophagosomes. We observed that autophagy at the synapse can be modulated in *vivo* by the state of neuronal activity, that autophagosomes undergo UNC-

16/JIP3-mediated retrograde transport, and that autophagosomes containing synaptic material mature in the cell body. Through forward genetic screens, we then determined that autophagosome maturation in the cell body depends on the protease ATG-4.2, but not the related ATG-4.1, and that ATG-4.2 can cleave LGG-1/Atg8/GABARAP from membranes. Our studies revealed that ATG-4.2 is specifically necessary for the maturation and clearance of autophagosomes and that defects in transport and ATG-4.2-mediated maturation genetically interact to enhance abnormal accumulation of autophagosomes in neurons

P480/B492

RNA-sequencing Identifies Altered Gene Expression in the Ventromedial Hypothalamus Following Acute Predator Odor Exposure.

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Energy expended during activity is the second largest contributor to our total daily energy expenditure (EE). Uncovering ways to increase EE during activity could yield new treatments for obesity. The ventromedial medial hypothalamus (VMH) regulates body weight by modulating peripheral metabolism and sympathetic nervous system (SNS) activity. Interestingly, the VMH is a critical mediator of behavioral responses to predator threat. Previously, we've shown that predator odor (PO) exposure to rats causes a rapid and robust increase in skeletal muscle thermogenesis and EE even when controlling for physical activity and is associated with weight loss. VMH cells are a likely mediator of these PO-induced metabolic responses, but the causal links remain unexplored. To probe potential targets, we performed RNA-sequencing on RNA isolated from the VMH of rats exposed to a piece of towel (2" x 1.5") previously housed with ferrets or a clean piece of towel in their homecage. After 20 min of exposure, rats were anesthetized with isoflurane and rapidly decapitated. Brains were flash frozen in isopentane on dry ice. RNA was isolated from VMH micropunches using a combination of Trizol and columns. Samples were sent to Novogene for RNA-sequencing and analysis. An alysis using DESeq software identified 164 differential expressed genes (DEGs). A gene ontology (GO) enrichment analysis revealed 65 enriched GO terms. Kyoto encyclopedia of genes and genomes (KEGG) analysis indicated enrichment of 28 pathways. Taken together, our results showed common themes of immune response, oxidative stress, alterations in inter-cell communication and neuronal morphogenesis and plasticity. Cross-examining our findings with previous literature revealed an interesting pattern wherein many of our DEGs and enriched pathways have independently been implicated in brain regulation of metabolism and/or response to predator threat, but rarely have these DEGs been functionally connected in these contexts. We are the first to explore transcriptome-wide alterations of the VMH following PO exposure. Our results have uncovered novel functional relationships among genes that can now be probed to better understand the dual processing role of the VMH in metabolism and predator threat responses.

P481/B493

Interplay between Nmda and Ampa Type Glutamate Receptors May Facilitate Learning in Caenorhabditis Elegans.

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In order for nerve cells to communicate with each other, they must use receptor proteins to send chemical and electrical signals. One class of receptor proteins that are central to learning and memory in

mammals are the glutamate receptors. Changes in protein and mRNA levels of glutamate receptors underlie the neurobiology of learning and memory. Glutamate receptors are also found in the model organism, *C. elegans*. In *C. elegans*, NMDA and AMPA type glutamate receptors, called NMR-1 and GLR-1 respectively, are involved in learning as well as movement. In order to understand how NMR-1 and GLR-1 cooperate in associative learning, we tested how behavior and biochemistry are affected when animals with the presence and absence of *nmr-1* are challenged to associate a stimulus with a tone. In order to assess changes in *C. elegans* during associative learning, animals were synchronized by age, then trained by subjecting them to two aversive stimuli: a vibrational and blue light stimulus. Behavioral testing revealed that after training, animals have a statistically significant change in behavioral response in wildtype and *nmr-1* worms. We also measured how GLR-1 tagged with GFP (GLR-1::GFP) changed in worms with a deletion in the *nmr-1* gene. We measured GLR-1::GFP protein abundance with western blotting and mRNA levels with qPCR (TaqMan) assays.A large increase in GLR-1::GFP mRNA, but a decrease in GLR-1::GFP protein, was seen in wild type worms after training. However, naïve and trained *nmr-1* mutants only showed a decrease in GLR-1::GFP protein abundance. These findings suggest there may be other proteins or compensatory mechanisms involved in regulating GLR-1 in both scenarios. Additional experiments are being carried out on all strains by collecting worms immediately after and 24hr after being trained establish whether the discrepancy between mRNA and protein levels is a time dependent event.

P482/B494

Cocaine-induced Functional Deficit in Orbitofrontal Cortex Is Prevented by Systemic Administration of a Sigma-1 Receptor an tagonist.

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The orbitofrontal cortex (OFC) is necessary for inferring expected outcomes to guide appropriate responding. This function can be shown in a sensory preconditioning task, in which behavior to the preconditioned but not the directly conditioned cue is sensitive to inactivation of the OFC after learning. Self-administration of cocaine causes similar deficits in preconditioning, suggesting drug-induced problems in model-based inference that might complicate treatment.The sigma-1 receptor (Sig-1R) is a chaperone protein predominantly expressed on the ER membrane. It has been reported that the receptor modulates the function of other proteins by physical interaction. Many synthetic compounds, including psychiatric drugs and psychostimulants bind to the Sig-1R, and cocaine is known to act as an agonist. We previously reported that a Sig-1R antagonist, BD1063, given prior to administration of cocaine, attenuated behavioral sensitization caused by cocaine, and reversed the cocaine-induced hypoexcitability of medium spiny neurons in nucleus accumbens. Here, we investigated the potential role of Sig-1R in cocaine-induced functional alterations of OFC. Sig-1Rs were localized in the rat OFC by immunohistochemistry and western blot. Rats were given the opportunity to spontaneously acquire self-administration of cocaine or sucrose, with a prior injection of either BD1063 or saline for 12 days. After four weeks of cocaine withdrawal, rats were trained in a sensory preconditioning task. As reported previously, rats withdrawn from cocaine self-administration exhibited a deficit in sensory preconditioning performance, failing to respond appropriately to the preconditioned cue. This deficit was not present in rats that had received the Sig-1R antagonist BD1063 prior to each cocaine self-administration session. The potential mechanisms underlying this result were further examined.

Unexpectedly, the electrophysiological activity of the serotonin 2A receptor (5HT-2AR) in the OFC of different treatment groups did not differ. An other potential target, the gamma-aminobutyric acid receptor (GABAR) which can interact with Sig-1R as seen per immunoprecipitation in HEK cells is now being investigated. Further, a relatively new tag, APEX2, combined with mass spectrometry analyses, is being used to reveal the sub-global profile of molecular changes among the groups. Although more experiments are needed including the use of Sig-1R antagonist during cocaine withdrawal, our data suggest the Sig-1R as a potential therapeutic target in the treatment of cocaine addiction. (This study supported by IRP/NIDA/NIH/DHHS)

P483/B495

Galectin-8, Target of Autoantibodies, Binds Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor (AMPAR) and Enhances Its Trafficking to the Cell Surface, Post-synaptic Transmission and Plasticity Involved in Spatial Memory.

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Galectin-8 (Gal-8) interacts with beta-galactosides moieties of selective cell surface glycoproteins including signaling receptors and integrins, with preference for alpha-2,3 sialic acids. In the brain, we have shown that Gal-8 has immunosuppressive and neuroprotective roles, being expressed in several brain regions, including hippocampus and the choroid plexus. Gal-8 is also present in cerebro-spinal fluid. Autoantibodies from patients with autoimmune diseases such as systemic lupus erythematosus (SLE) and multiple sclerosis can block Gal-8 functions. As these autoantibodies have been detected in the brain, it is important to understand the role of Gal-8 in neurons. We first performed a proteomic analysis of interactors, which pointed to AMPAR among other synaptic proteins. Pull-down and co-immunoprecipitation experiments confirmed Gal-8/AMPAR interaction involving alpha-2,3 sialic acids. AMPAR endocytic recycling is highly regulated and crucial in synaptic plasticity and memory processes. Surface biotinylation revealed that neurons in primary culture respond to Gal-8 by rapidly (30 min) increasing the surface levels of AMPAR, achieving a plateau at 1h, without altering endocytosis or total receptor mass, thus suggesting enhanced recycling. Even though protein kinase a (PKA) and N-methyl-D-aspartate receptor activity are known to increase post-synaptic levels of AMPAR, only PKA inhibitors decreased this effect. Focal Adhesion Kinase (FAK) inhibition also counteracted this effect, thus involving integrin activation. Electrophysiology assessing field excitatory post-synaptic potential and patch-clamp currents showed that Gal-8 increases AMPAR-mediated transmission. In concordance, Gal-8 knock-out mice displayed lower AMPAR levels on post-synaptic densities and impaired long-term potentiation (LTP) in hippocampal CA3-CA1 synaptic transmission, correlating with poor performance in spatial memory tests. Human anti-Gal-8 autoantibodies isolated from SLE patients alter LTP. All these results posit Gal-8 as a new modulator of AMPA receptor function, likely acting through integrins and PKA activation and impinging upon synaptic transmission and spatial memory. Autoantibodies that counteract Gal-8 function on glutamatergic transmission can potentially mediate cognitive dysfunction. (CONICYT Basal grant AFB170005. FWIS UNESCO-L'Oreal fellowship (MFB))

P484/B496

Parallel Processing of Two Mechanosensory Modalities by a Single Neuron in *C. Elegans*.

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Neurons convert synaptic or sensory inputs into cellular outputs. It is not well understood how a single neuron senses, processes multiple stimuli, and generates distinct neuronal outcomes. Here we describe the mechanism by which the *C. elegans* PVD neurons sense two mechanical stimuli: external touch and proprioceptive body movement. These two stimuli are detected by distinct mechanosensitive DEG/ENaC/ASIC channels, which trigger distinct cellular outputs linked to mechanonociception and proprioception. Mechanonociception depends on DEGT-1 and activates PVD's downstream command interneurons through its axon, while proprioception depends on DEL-1, UNC-8 and MEC-10 to induce local dendritic Ca^{2+} increase and dendritic release of a neuropeptide NLP-12. NLP-12 directly modulates neuromuscular junction activity through the Cholecystokinin receptor homolog on motor axons, setting muscle tone and movement vigor. Hence, the same neuron simultaneously uses both its axon and dendrites as output apparatus to drive distinct sensorimotor outcomes.

Neuronal Cytoskeleton

P485/B497

VASP Ubiquitination Regulates Actin Dynamics and Neuronal Morphogenesis.

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Filopodia are dynamic, actin-rich structures that extend outward from the cell and explore the local environment. In neurons, filopodia are critical for numerous stages of development, including neuritogenesis, axon guidance, and dendritic spine formation. Defects in any of these stages of neuronal development can result in improper synaptic connectivity, neurodevelopmental disorders, and psychiatric syndromes. The Ena/VASP of actin polymerases is well appreciated to localize to the filopodial tip complex and influence actin dynamics. Recently, the Gupton lab showed VASP transiently co-localizes with the E3 ubiquitin ligase TRIM9 at the filopodial tip. TRIM9 was required for the reversible, non-degradative ubiquitination of VASP and this modification was associated with decreases in filopodia stability and number at the growth cone. Although the dynamic actin cytoskeleton and VASP are also appreciated to play important roles in the postsynapse, it is not known how VASP activity is regulated in dendritic filopodia and the maturing dendritic spine. Here we show that VASP, TRIM9 and ubiquitinated VASP (VASP-Ub) localize to the PSD following differential centrifugation, suggesting a role for VASP-Ub in dendritic spines. Cultured murine cortical neurons lacking *Trim9* exhibit increased dendritic filopodia number at DIV7. Likewise, overexpression of VASP and a non-ubiquitinatable form of VASP (VASP-KR) also increase dendritic filopodia at DIV7. Based on these results, we hypothesize VASP-Ub is required for the regulation of actin dynamics during synaptogenesis. Ongoing work is examining *Trim9* deletion, as well as VASP and VASP-KR overexpression, on dendritic spine number and synaptic plasticity. Future work will explore the mechanistic impact of ubiquitination on actin-VASP interactions through in *vitro* biochemical reconstitution assays.

P486/B498

Neurofilament Transport Is Bidirectional in *Vivo* and Is Developmentally Regulated.

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The postnatal development of long axons is characterized by an accumulation of space-filling cytoskeletal polymers called neurofilaments, which drives the expansion of axon caliber necessary for rapid electrical transmission. Studies in cultured nerve cells using fluorescence microscopy have shown that neurofilament polymers move bidirectionally along axons in a rapid intermittent manner and that they can spend as much as half their time moving backwards. However, it is unclear whether neurofilament movement is also bidirectional in *vivo*. To test this, we developed a pulse-spread assay which measures the rate of spreading of a population of photoactivated fluorescent axonal neurofilaments. Tibial and sciatic nerve segments were dissected from Thy1-PAGFP-NFM transgenic mice, which express PAGFP-tagged neurofilament protein M in neurons, and imaged in a perfusion chamber. The filaments were photoactivated in a short segment of an axon and the increase in the fluorescence of the proximal and distal flanking regions due to the movement of the fluorescent neurofilaments was measured over time. Using computational modeling, we show that the difference of the slopes of the fluorescence in the flanking regions yields the neurofilament transport velocity and that the ratio of the proximal and distal slopes yields the directional bias. Our data demonstrate active bidirectional transport of neurofilaments in myelinated axons from mice at 2, 4, 8 and 16 weeks of age, with a substantial fraction moving retrogradely. The neurofilament transport rate decreased with age, which is consistent with previous reports obtained using radioisotopic pulse labeling. Approximately 55% of the transport was anterograde in mice aged 2, 4, and 8 weeks, while no directional bias was detected at 16 weeks. Thus, we conclude that neurofilament transport is bidirectional in *vivo and* that the filaments spend a significant fraction of their time moving retrogradely. The lack of directional bias at 16 weeks suggests that there is no net movement of the polymers in distal axons of older animals. It is intriguing that axons invest metabolic energy to move neurofilaments backwards, and it suggests that neurofilament transport does not function only to deliver neurofilaments to distal regions of axons. It is possible that the retrograde movement is important for neurofilament turnover or degradation, but we favor the idea that the bidirectional movement of these polymers may function more to distribute and/or organize these polymers.

P487/B499

Neurite Morphogenesis Requires Suppression of the Lamellar Protrusions of the Cell Body by Septin 7.

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Axons and dendrites, the structures that transmit and receive electrical and chemical signals in the brain, develop from the membrane protrusions of neuronal cell bodies. Formation of neurites, the early precursors of axons and dendrites, involves the formation of actin-rich filopodia and their engorgement with microtubules. How actin and microtubules are coordinated and how neurites mature and grow asymmetrically into axons and dendrites is not well understood. Septins are a family of filamentous GTP-binding proteins, which associate with actin and microtubules, and septin 7 (Sept7) was recently found to determine the sites of neurite formation in neuronal progenitors. Here, we have sought to investigate the role of septins in early neurite formation and asymmetry establishment. We found that that Sept7 depletion in embryonic rat hippocampal neurons (DIV1) resulted in neurons with abnormally enlarged

cell bodies, which lacked pyramidal-shaped morphology and characterized by extensive lamellipodia-like protrusions. Strikingly, several neurites were observed to extend directly from these lamellipodial protrusions rather than directly from the cell body. This phenotype was accompanied by a reduction in the number of neurons that broke neurite symmetry and developed a single elongated tau-positive neurite, the presumptive axon. Expression of the constitutively active myosin II regulatory light chain in Sept7-depleted cells rescued partially the size and shape of neuronal cell bodies, and restored neurite formation from the cell body as well as axonal specification. Taken together, our data suggest that Sept7 functions synergistically with myosin-II in suppressing membrane protrusive activity and possibly branched actin polymerization, restricting neurite growth and maturation to filopodia that are in direct continuum with the cell body.

P488/B500

Doublecortin Supports Growth Cone Microtubule Function in Early Neuronal Morphogenesis.

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Numerous familial and spontaneous mutations in Doublecortin (DCX), a microtubule-associated protein highly expressed in the developing nervous system, cause a spectrum of debilitating neurodevelopmental conditions, such as X-linked Lissencephaly or Subcortical Band Heteropia. It is thought that DCX mutations disrupt the migration of immature neurons through the developing cortex. However, the molecular role of DCX in early neuronal development and how DCX controls organization and function of the neuronal microtubule cytoskeleton is not well understood and is mostly based on in vitro experiments or overexpression of DCX in non-neuronal cells. To directly investigate DCX function and dynamics at endogenous expression levels in developing neurons, we fluorescently tagged the C-terminus of the endogenous DCX locus in human induced pluripotent stem cells (iPSCs) by CRISPR/Cas9 genome editing. Developing DCX-eGFP iPSC-derived neurons show a similar increase of DCX expression as control cells, and time-lapse microscopy of DCX-eGFP confirms our previous finding of DCX-eGFP specificity for straight GDP-lattice microtubules. Notably, we never observe DCX-eGFP tracking of growing microtubule ends and never observe bundling of DCX-eGFP labeled microtubules. However, we find that DCX-eGFP only binds to microtubules in neuronal growth cones or nascent branches along the neurite shaft. Inhibition of DCX-microtubule binding in the neurite shaft may be due to phosphorylation, as pharmacological inhibition of glycogen synthase kinase 3 (GSK3) increased DCX localization to neurite microtubules. We previously found that taxanes reverse the microtubule geometry specificity of DCX. Based on experiments in which taxanes induce characteristic bending of neuronal growth cone microtubules, we hypothesize that DCX functions to straighten and/or stiffen microtubules in advancing growth cones. To directly test this hypothesis and test at a molecular level how disease-associated DCX mutations impede nervous system development, we are now modeling clinical DCX mutations in our iPSC neuronal differentiation system.

P489/B501

Modifying Levels of Acetylated Microtubules in Growth Cones Triggers Redistribution of Eb3.

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In the peripheral domain of growth cones, interactions between F-actin and dynamic microtubules influence growth cone behavior and direction of axon advance. Our previous research showed an

inverse relationship between levels of microtubule acetylation and microtubule invasion into the peripheral domain, but the mechanism of why this occurs is not known. EB3, a microtubule plus-end tracking protein, plays an important role in promoting the interaction between dynamic microtubules and F-Actin in the peripheral domain of growth cones. Here, we tested if modifying the levels of microtubule acetylation would affect the localization of EB3 in growth cones. To increase the amount of acetylated microtubules (AcMTs), we used tubacin, a cell-permeable inhibitor of HDAC6, an enzyme that removes acetyl groups from MTs. To decrease the amount of AcMTs, we used resveratrol, an activator of sirtuin, an enzyme that de-acetylates microtubules. Following exposure to either tubacin or resveratrol, *Helisoma* neurons were immunostained with antibodies for EB3 and tubulin. Quantification of fluorescent intensity in neurons exposed to tubacin showed a significant increase in the amount of EB3 present in the filopodia compared to control cells, while neurons exposed to resveratrol showed a significant decrease in the amount of EB3 present in the filopodia compared to control cells. Additionally, threshold analysis of EB3 fluorescence in the peripheral domain revealed that as the levels of AcMTs increased, the levels of EB3 decreased. These results suggest that microtubule acetylation promotes redistribution of EB3, resulting in suppression of EB3 interactions with microtubules.

P490/B502

CB1R and Myosin II Function in Regulation of Growth Cone Protrusions and Axon Projections in the Optic Tract.

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A key step in the formation of neuronal circuits involves axons navigating through diverse regions of the brain to their target tissue. In the developing retino-tectal projection of lower vertebrates, optic axons extend through the optic tract towards the optic tectum in the midbrain. Here, we studied how the main cannabinoid receptor in the brain- CB1R- and the actin regulator Myosin II modulate optic axon pathfinding and growth cone filopodia in the optic tract in brains of *Xenopus* tadpoles. Embryos and tadpoles containing GFP optic axons were bathed in chemical inhibitors specific for CB1R (AM251) or Myosin II (Blebbistatin) during developmental stages when optic axons normally extend through the optic tract. The tadpoles were then fixed and their brains were dissected, and the optic axons were imaged in the optic tract of whole brains. These experiments showed that AM251 increased dispersion and induced aberrant turning of optic axons away from the optic tectum. In contrast, Blebbistatin inhibited the extension of optic axons through the optic tract. However, optic axons from tadpoles exposed to both the CB1R and Myosin II inhibitors formed growth cones with increased numbers of filopodial protrusions. These results suggest that CB1R and Myosin II exert both shared and distinct functions in regulating growth cone filopodia *and* optic axonal projections *in* the optic tract. More broadly, our findings imply that optic axonal growth cones may express distinct types of filopodia that regulate specific axon pathfinding behaviors such as fasciculation and extension.

P491/B503

A Mechanism for Neurofilament Transport Acceleration through Nodes of Ranvier.

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Neurofilaments are abundant space-filling cytoskeletal polymers in axons that are transported along microtubule tracks. Experiments show that neurofilament transport is accelerated at nodes of Ranvier,

where axons are locally constricted. Strikingly, these constrictions are accompanied by a sharp decrease in neurofilament number but no decrease in microtubule number, bringing neurofilaments closer to their microtubule tracks. In this work, we test the hypothesis that improved access to microtubules leads to an increase in the proportion of the time that the filaments spend moving and that this can explain the local acceleration. We developed a stochastic model of neurofilament transport that takes into account neurofilament length distribution and tracks their number, kinetic state and proximity to nearby microtubules in space and time. The model assumes that the probability of a neurofilament actively moving is dependent on its distance from the nearest available microtubule track. We use experimentally reported numbers and densities for neurofilaments and microtubules in axonal nodes and internodes and show that this model is sufficient to explain the local acceleration of neurofilaments across nodes of Ranvier. The proposed modeling framework can be used to predict and illustrate the impact of filament length distributions and neurofilament to microtubule ratios on the ability of neurofilaments to navigate these physiologically important axonal constrictions. Our results suggest that proximity to microtubule tracks may be a key regulator of neurofilament transport in axons, which has implications for the mechanism of neurofilament accumulation in development and disease.

P492/B504

Kinetochore Protein Spindly Controls Microtubule Polarity in Axons by Recruiting Dynein to F-actin.

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Polarity of microtubules in axons and dendrites defines intracellular transport in neurons. Axons contain uniform microtubule arrays with plus-ends facing the tips of the processes, while dendrites contain microtubules with a minus-end-out orientation. We have recently demonstrated that cytoplasmic Dynein, targeted to cortical actin, removes minus-end-out microtubules from axons. Here we have identified Spindly, a protein who's known role is to recruit Dynein to the kinetochore in mitosis, as a key factor of in Dynein-dependent sorting of microtubules in axons of *Drosophila* neurons. Depletion of Spindly affects polarity of axonal microtubules in *vivo* and in primary neuronal cultures. In addition to axonal microtubule polarity defects, depletion of Spindly in neurons causes major collapse of axonal patterning in third instar larvae brains as well as dramatic locomotion and coordination impairment in adult flies. These phenotypes can be fully rescued by ectopic expression of wild-type Spindly, but not by variants with the mutation in the dynein-binding site. Biochemical analysis demonstrated that Spindly binds F-actin, confirming that Spindly serves as a link between Dynein and cortical actin in axons. Thus, a "mitotic" protein Spindly plays a critical role during neurodevelopment by facilitating Dynein-driven sorting of axonal microtubules. Supported by NIGMS grants GM-052111 and GM-131752 to VIG

P493/B505

Radial Axonal Growth and Slowing of Neurofilament Transport.

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The radial growth of axons during postnatal development is an important influence on axonal conduction velocity. This axonal expansion is driven in part by the accumulation of space-filling

cytoskeletal polymers called neurofilaments. Neurofilaments are delivered to axons by molecular motors, which transport the filaments rapidly but infrequently along microtubule tracks. The filaments move forwards and backwards, but with an anterograde bias, resulting in a slow net velocity of 0.1-2 mm/day towards the axon tip. Experimental studies have shown that the neurofilament accumulation is accompanied by an increase in neurofilament expression, which suggests an increase in neurofilament flux from the cell body, and a slowing of neurofilament transport velocity within the axon. We have used computational modeling to investigate the cause of this slowing and its contribution to radial axon growth. In our model, neurofilaments enter the axon proximally and move along microtubules, cycling through kinetic states in which they move either anterogradely or retrogradely or pause. We consider that the neurofilaments are radially mobile and that they must make a diffusive encounter with a microtubule in order to move. Thus, the probability of moving is inversely proportional to the average distance between the neurofilament and its nearest microtubule. To model radial growth, we adjusted the influx of neurofilaments, which has not been directly measured, to match the observed neurofilament content and axonal caliber during the postnatal development of the rat sciatic nerve. We increased the number of microtubule tracks according to published morphometric data. We then used this model to simulate the movement of a pulse of radiolabeled neurofilaments along the axon at different stages of postnatal development. We found that initially the velocity of the pulse is much faster than the average velocity of the overall neurofilament population regardless of whether there is radial growth. The pulse slows down to the average neurofilament velocity within a few days. We also found that the slowing of the average neurofilament velocity during postnatal development emerges in the model because the increase in neurofilaments outpaces the increase in microtubules, increasing the average distance of the neurofilaments from their nearest microtubule track. Our analysis indicates that the growth and maintenance of axon caliber is a complex interplay of neurofilament flux from the cell body and transport velocity within the axon. The model permits us to estimate the relative contribution of an increase in neurofilament flux and a decrease in neurofilament transport velocity to the growth of axon caliber.

P494/B506

Local Regulation of Microtubule Organization and Synaptic Cargo Delivery by an Immotile Kinesin.

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Acentrsomal microtubule arrays support long-range transport of neuronal cargo. How properties of neuronal microtubules - array density, polymer length, dynamics and distribution - are regulated, and how they affect the delivery of synaptic cargo, is poorly understood. We established an experimental system to examine microtubule organization, dynamics and cargo delivery in *vivo*, with single-cell resolution in living *C. elegans* nematodes. From an unbiased genetic screen, we identified the immotile kinesin VAB-8/KIF26 as a regulator of neuronal microtubule organization. *vab-8* mutants show specific and local microtubule organization defects at proximal presynaptic varicosities, including reduced polymer numbers and increased polymer length. Surprisingly, axonal transport does not stall on these abnormal tracks, but vesicles instead accumulate in more distal presynaptic sites, suggesting a specific failure in synaptic cargo delivery. VAB-8/KIF26 is known to regulate cell migration downstream of Wnt signaling. We found that in the context of patterning synaptic connectivity, VAB-8/KIF26 also acts downstream of the Frizzled receptor, and identified mutations in a Frizzled receptor E3 ligase, PLR-1/RNF43 that lead to identical microtubule and transport defects as *vab-8*. We propose that local

regulation of acentrosomal microtubules by extracellular cues ensures robust cargo delivery and patterning of synaptic connectivity.

P495/B507

Neuron Development and Functioning Depends on the Proper Microtubule Organization; a Balancing Act between Sliding and an choring.

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The proper organization of the microtubule cytoskeleton is essential for neuronal development and survival. The microtubules serve as tracks for long range cargo transport and enable for selective transport into the axon and dendrites to establish neuronal polarity. In addition, the regulation of microtubule integrity is tightly linked to neuron survival and regeneration. Even though crucial for proper neuronal functioning, the molecular mechanisms that set-up and maintain the microtubule organization are for a large part unclear. Here, we use *Caenorhabditis elegans* as an in *vivo* model system to study neuron development and found that UNC-119 (homologous to human UNC119A and UNC119B) is essential microtubule organization in axons and dendrites. By binding to the cortical UNC-44 (Ankyrin) and the microtubule binding UNC-33 (CRMP), UNC-119 forms a periodic membrane-associated cytoskeletal complex that anchors the plasma membrane to the underlying microtubule cytoskeleton. In the absence of either of these proteins, the microtubule cytoskeleton shows dramatic sliding and becomes disorganized, resulting in axon-dendrite polarity defects. This sliding is induced by the motor protein UNC-116 (kinesin-1), which in wildtype animals is essential for dendritic microtubule organization. We propose that the proper development of axons and dendrites relies on a balance between motor induced microtubule sliding and anchoring to the plasma membrane.

P496/B508

Growing Tip-localized Microtubule Organizing Center Determines Microtubule Orientation in Dendrites.

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Neuronal subcellular compartmentalization of axon and dendrite provides a directionality that is essential for informational flow through the nervous system, and how it is established remains unclear. By observing the development of a single highly polarized neuron from its birth in *C. elegans*, we find it polarizes and outgrows its axonal and dendritic neurites with highly stereotyped orientation and sequence. The unique behavior of each neurite even as it first emerges suggests that the establishment of subcellular compartmentalization is concomitant with neurite emergence in *vivo* rather than a delayed neurite maturation process as is seen in cultured neurons removed from their in *vivo* environment. As tracks for directional subcellular transport, a polarized arrangement of microtubule arrays lays the foundation for axon versus dendrite compartmentalization. Mature axons are characterized by exclusively “plus-end-out” microtubules while dendrites uniquely contain a large population of “minus-end-out” microtubules. Here we identify a mechanism by which the characteristic minus-end-out microtubule orientation is initially established in dendrites. By observing microtubule plus-end growth during dendrite emergence and outgrowth, we find a microtubule-organizing center

(MTOC) that localizes near the distal dendrite tip as it emerges and continues to advance. This MTOC generates numerous minus-end-out microtubules that populate the dendrite. Core components of the gamma tubulin ring complex localize to the dendritic MTOC. Cell-specific degradation of these components indicates they are required for the dendritic MTOC and establishment of the minus-end-out microtubules characteristic of mature dendrites. We continue to characterize the subcellular nature, regulation, and positioning of this growing tip-localized dendritic MTOC.

P497/B509

Laser-induced Severing Reveals Evidence for a Preferential Association of Motors with the Leading Ends of Moving Neurofilaments.

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Neurofilaments are long flexible cytoskeletal polymers that are transported rapidly and intermittently along axonal microtubule tracks powered by kinesin and dynein motor proteins. Time-lapse and kymograph analyses of neurofilaments in axons of cultured neurons have revealed that these polymers often fold and unfold reversibly, however, they almost always become fully extended when they move. To explain this behavior, we hypothesize that neurofilaments are pulled from their leading ends, which implies that neurofilament motors associate preferentially with the leading ends of these polymers. To test this hypothesis, we used a nanosecond-pulsed dye laser to sever single moving neurofilaments in axons and examined the fate of the severed fragments. If motors associate exclusively with the leading end of the moving filaments then we would expect that the leading fragment would continue to move and the trailing fragment would not. Neurofilaments in primary cultures of rat cortical neurons were transfected with GFP-tagged neurofilament protein L to label neurofilament polymers and the movement of the polymers was tracked by time-lapse imaging with 100 ms exposures and 2-3 second time intervals. Moving neurofilaments were irradiated on the fly by 1-5 nanosecond pulses of 435 nm light delivered using an an dor MicroPoint laser. The GFP fluorescence on the filament was bleached immediately, marking the site of irradiation. By attenuating the intensity of the laser, we were able to induce delayed severing, permitting the filaments to move away from the original axonal location of irradiation before they severed. In this way, we were able to avoid any concern about possible damage to the axon or microtubule tracks at the location of filament severing. After severing, we tracked the fate of the filament fragments. 58% of the time (26/45 filaments) only the leading end continued to move and the trailing end did not. 20% of the time (9/45 filaments) only the trailing end moved and the leading end did not. 22% of the time (10/45 filaments) both the leading and trailing ends moved. Interestingly, when the leading end moved, 92% of the time (33/36 filaments) it continued to move in the same direction as the filament was moving before it severed, but when the trailing end moved, 73% (12/19) of the time it moved in the opposite direction that the filament had been moving before it severed. These observations suggest that (1) motors associate preferentially, but not exclusively, with the leading ends of moving neurofilaments, (2) there can be multiple sites of motor attachment along the moving filaments, and (3) motors of opposite directionality can associate with the same moving filament, sometimes at opposite ends.

P498/B510

Spatiotemporal Optogenetic Control of Microtubule Dynamics during Neuronal Morphogenesis.

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Neurons are amongst the most highly polarized and elongated cell types found in animals, and this morphology depends on the proper regulation of the microtubule (MT) cytoskeleton which provides structural support, and forms an intracellular railway system required to generate and sustain polarity. MTs are critical for many aspects of neuronal morphogenesis, such as neurite elongation, branching and neuronal navigation. MTs, while very rigid structures, are also highly dynamic, in order to support rapid reorganization and morphological plasticity. Directional navigation of neurons is mediated by the neuronal growth cone which senses and responds to chemotactic guidance cues, and this process depends on dynamic pioneering MTs in the growth cone periphery that polarize in the direction of migration. While it was demonstrated that this is related to the activation of Rho GTPases (key regulators of the actin cytoskeleton), it remains unclear how local regulation of MT dynamics controls growth cone steering. To gain a better understanding of this process, we have generated iPSC-derived neurons in which we can spatially control the activity of an important regulator of MT dynamics (the +TIP EB1) by light. We accomplished this via a single step genome editing strategy, that allowed us to directly insert a photo-inactivation element into the EB1 gene. We demonstrate that the growth rate of MTs in pi-EB1 knock-in iPSCs is not significantly different from WT cells in the dark, but decreases upon blue light inactivation of pi-EB1, similar to our previous results in other cell types. pi-EB1 expressing iPS cells could be efficiently differentiated into glutamatergic neurons, and allowed us to locally inactivate the +TIP complex in neuronal growth cones by using patterned blue light illumination. By confining the growth of these neurons to precisely controllable adhesion patterns we are now able to test the role of MT dynamics during neuronal growth cone migration in a highly standardized manner.

P499/B511

Unique β-spectrin N-terminus Is Required for Actin Binding and Neuronal Morphogenesis.

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Beta-spectrin binds actin to form a sub-plasma membrane cytoskeleton that is required for neuronal morphogenesis. We recently demonstrated that binding of beta-spectrin to actin is enhanced 1000-fold by a spinocerebellar ataxia type 5 (SCA5) L253P mutation that opens the interface between the tandem calponin homology (CH) subdomains comprising the ABD, allowing CH1 to directly bind actin. In addition, we identified a unique N-terminal sequence, preceding the conserved ABD, which also binds actin. The objective of the current study is to determine the functional requirement of the N-terminus for actin binding and beta-spectrin function in neurons. Using in vitro binding assays, we found that the high actin affinity of the mutant L253P ABD is abolished by N-terminal truncation. Thus, the N-terminus is required for actin binding even in the presence of a mutation that induces opening of the CH interface. To test the function of the N-terminus in vivo, we generated new Drosophila lines expressing beta-spectrin transgenes with N-terminal truncations (delta N). Pan-neuronal expression of delta-N-beta-spectrin causes toxicity. In contrast, N-terminal truncation eliminates toxicity caused by L253P mutation, consistent with a function of the N-terminus to modulate actin binding. To examine functional requirement of the N-terminus in neuronal morphogenesis, beta-spectrin transgenes were expressed

specifically in peripheral class IV da neurons. Delta-N-beta-spectrin causes severe loss of dendritic arborization, consistent with a toxic function of the truncated protein. In contrast, N-terminal truncation rescues arborization defects caused by the L253P mutation. In conclusion, our data suggest that opening of the CH1-CH2 interface is not sufficient to induce actin binding. Instead, the binding mechanism requires the unique N-terminus. Further, the N-terminus is essential for spectrin function in vivo to support formation of complex dendritic arbors.

P500/B512

A Charcot-Marie-Tooth Disease Type 2E Point Mutation in Neurofilament Protein L (NFL) Causes Altered Neurofilament Protein Levels and Impaired Neurofilament Assembly in Vivo.

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Neurofilaments are neuron-specific cytoskeletal polymers that contribute to axon expansion during development. The five subunits of neurofilaments are NFL, NFM, NFH, α-internexin, and peripherin. Charcot-Marie-Tooth disease type 2E (CMT2E) is a slowly progressive peripheral neuropathy of unknown mechanism caused by mutations in the gene that encodes NFL. The $NFL^{N98S/+}$ CMT2E mouse model, which is heterozygous for the N98S mutation in the endogenous mouse Nefl locus, has a disease phenotype and offers an opportunity to study the effects of a CMT2E mutation on neurofilaments in vivo. With this mouse we can ask several questions: 1) Is the mutant protein stably expressed? 2) Is the mutant protein incorporated into filaments? 3) Are the expression levels and assembly of the other neurofilament subunits altered? Protein quantification in spinal cord by Western blotting revealed a moderate reduction of NFL, NFM and α-internexin, a moderate increase of NFH, and a marked increase of peripherin. In contrast, quantification in the sciatic nerve revealed a marked reduction of NFL, NFM, NFH and α-internexin and a lesser reduction of peripherin. Using tandem mass spectrometry of tryptic digests, we determined that 41% of the NFL protein in the spinal cord was the N98S mutant protein. To determine the assembly state of the neurofilament proteins, we homogenized spinal cord tissue in Triton X-100 and performed centrifugation to separate soluble (unassembled) and insoluble (assembled) fractions. In the mutant, we observe an elevation of neurofilament proteins in the Triton-soluble fraction and a reduction in the Triton-insoluble fraction. On average, 41% of the NFL protein in the soluble fraction was mutant compared to 30% in the insoluble fraction. Electron microscopy of spinal cord, dorsal root ganglia and sciatic nerves confirmed that most myelinated axons lacked neurofilaments and exhibited reduced caliber, but some axons in the dorsal root ganglia and spinal cord were enlarged and contained neurofilament accumulations. Our data suggest that expression of the mutant NFL results in an impairment of neurofilament assembly in the spinal cord and a profound loss of neurofilament proteins and neurofilaments in peripheral nerve myelinated axons. The loss of NFL protein in the spinal cord is due to a loss of both mutant and wild type protein. Thus, there does not seem to be a selective destabilization of the mutant protein. We hypothesize that disease may arise from a failure of neurofilaments to enter axons due to sequestration and destabilization of neurofilaments in cell bodies and proximal axons caused by either (1) alterations in the structure or subunit stoichiometry of the filaments, or (2) toxicity or gain-of-function of elevated soluble neurofilament protein.

Establishing and Maintaining an Organelle 1

P501/B514

The Cell Atlas in the Human Protein Atlas: an Image-based Subcellular Map of the Human Proteome.

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The spatial division into organelles and other subcellular structures is the defining principle of the eukaryotic cell. This concept allows multiple reactions to occur in parallel in an optimized and often enclosed environment. Each of these organelles has its own proteome for fulfilling the respective function at a specific time and place. Hence, knowledge of the subcellular localization of a protein is an essential step in its characterization and can provide evidence about potential functions and interactions. The publicly available Cell Atlas, part of the Human Protein Atlas (HPA) database, is a valuable resource designed for researchers that are interested in the spatial distribution of proteins. We generated the most comprehensive map of human protein distribution, which is based on ten thousands of immunofluorescent images. For that, we used the large HPA antibody collection, which contains antibodies targeting all human proteins. The updated HPA Cell Atlas covers now 12,390 proteins mapped to 33 subcellular locations, enabling the definition of 13 major organelle proteomes. The high spatial resolution of the IF images enabled the identification of novel protein components of fine structures such as the midbody, nuclear bodies or rods and rings. The image data is complemented by RNA sequencing data for 64 cell lines of various tissue origins, connecting gene expression and cell line-specific characteristics with subcellular localization. One major finding was the discovery of many new multilocalizing proteins (MLPs). MLPs are proteins which are detected at more than one organelle. Approximately half of the analyzed proteins were MLPs (6647), with 29% of them (1960) at three or more locations, confirming that multilocalization is a common feature of the human proteome. An other key feature of the HPA Cell Atlas is the possibility to look at single cell variations. About a fifth of the analyzed proteins showed spatial or quantitative variations in the protein expression and further analysis demonstrated that only a subset of variations could be linked to the cell cycle. Furthermore, we implemented the localization data from the HPA Cell Atlas to a protein-protein interaction network and thereby improved its quality. This indicates that other network analyses could benefit comparably. Finally, the IF images have been successfully used in the training of various models for image classification, underlining the high quality of images. The HPA Cell Atlas provides in combination with the other HPA sub-atlases for humantissues, brain, blood and cancer a holistic view on the human proteome in health and disease.

P502/B515

The Properties of Membraneless Organelles Are Tuned to Environmental Conditions.

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How cells pattern and compartmentalize their cytoplasm is a critical question in biology. Many cells rely on membraneless organelles for organizing biochemistry. These organelles are not separated from the rest of the cell by a lipid bilayer, instead they are formed through Liquid-Liquid Phase Separation (LLPS) in which a well-mixed solution demixes into a concentrated droplet phase and a dilute bulk phase. When the material properties, such as viscosity, of these droplets are measured they are found to vary by a thousand fold. Despite these differences we have very little understanding about whether specific properties are important for the function of droplets, although it has been suggested that dysregulation of droplet properties can result in diseases. In the free-living filamentous fungus *Ashbya gossypii* asynchronous nuclear division in the syncytial hyphae requires the phase separation of the protein Whi3 and the cyclin RNA *CLN3*. Whi3 also undergoes phase separation with the polarity RNAs *BNI1* and *SPA2*, which is required for hyphal branching. Like other phase-separating proteins Whi3 contains an RNA recognition motif (RRM) that allows for multivalent interactions, and it contains a disordered domain rich in glutamine (polyQ). Loss of either of these domains results in failure to phase separate. Because LLPS is a thermodynamically-driven process, temperature plays a role in determining whether phase separation happens and influences the properties of the resulting droplets. Free living fungi are exposed to the ambient temperature and therefore are under selective pressure to adapt to their local climate. We hypothesized that if the material properties of droplets are important then they should be optimized for a given climate. Using a collection of fifty wild-isolated *Ashbya* from different climates in the United States we sought to test the effects of temperature on the properties of membraneless organelles. We found that the interbranch distance, which is controlled by membraneless organelles, varied between isolates when grown at high or low temperature. We then performed whole genome sequencing on the wild isolates. We found that the sequence of the Whi3 polyQ domain, which is critical for phase separation, differs between isolates and that purified proteins from these isolates behaves differently in *in vitro* phase separation experiments. We further found that the sequence of the RNA of *CLN3* and *BNI1* differs between isolates and that these changes affect the structure of those RNAs without changing the protein coding sequence. These results suggest that the material properties of phase separated droplets are tuned to the climate at which the cells live indicating that specific properties are important for function.

P503/B516

Coupled Oscillators Self-organise the Assembly of Centrioles.

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The regulation of organelle biogenesis is a vital task. Centrioles are membraneless organelles, comprising a central cartwheel surrounded by microtubule (MT) blades. During centriole biogenesis, the cartwheel and MTs grow from opposite ends of the organelle, but ultimately reach the same size/ratio.

This study aims to address how this is achieved. We previously identified an autonomous Polo-like kinase 4 (Plk4) oscillator concentrated at the base of growing daughter centrioles, which times and executes cartwheel growth from this proximal-end. Here, we discover a CP110/Cep97 protein complex forms a separate autonomous oscillator at the tip of the daughter centriole, which appears to time the growth of the centriolar MTs at the distal-end. Remarkably, although spatially separated, the Plk4 and CP110/Cep97 oscillators are temporally coupled, ensuring the coordinated growth of the cartwheel and MTs. Coupled oscillators provide a novel, powerful mechanism for timing and coordinating the assembly of this complex organelle and might represent a more general feature of organelle biogenesis.

P505/B518

Testing the Constancy of the Nuclear to Cell (N/C) Volume Ratio in Chlamydomonas.

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Maintenance of a constant nuclear to cell volume ratio (N/C) is a conserved trait in eukaryotic cells, though the absolute N/C ratio varies by species and by cell type. The green alga *Chlamydomonas reinhardtii* (Chlamydomonas) uses a multiple fission mitotic cell cycle where cells have a long G1 phase, during which they can grow more than ten-fold in size. G1 is followed by rapid alternating rounds of DNA syntheses and mitosis (S/M) to produce a uniform-sized population of new daughters. We took advantage of the large cell-size range observed in synchronized Chlamydomonas cultures to test the limits of N/C across different cell sizes. N/C was tracked in live cells using a nuclear-localized ble-GFP fusion protein as an in *vivo* marker for nuclei. We found that N/C remains nearly constant at around 5% across the cell cycle in wild-type haploid and diploid cells, and in cell-size mutants. By comparison, the shoot meristem cells and epidermal cells of Arabidopsis have an N/C ratio of 20%. The 135 Mb Arabidopsis genome is only slightly larger than that of Chlamydomonas (120 Mb) indicating that genome size is not the major parameter which sets N/C. We also observed a distortion of N/C in the smallest Chlamydomonas cells suggesting that there may be a lower limit of N/C constrained by the physical packing of DNA. Tests of the lower limit for N/C in diploid cells, which was higher than in haploid cells, supported the idea that N/C becomes physically constrained by nuclear DNA content in very small cells.

P506/B519

The Role of Osmotic Forces in Nuclear Size Control In*S.pombe*.

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The size of the nucleus scales with cell size so that the nuclear-to-cell volume ratio (NC Ratio) is maintained during cell growth. The mechanism responsible for this scaling is unknown. Nuclear volume is not determined merely by DNA amount, but is influenced by factors such as the surface area of the nuclear envelope, nuclear transport and osmotic pressure. Very little is known about mechanical basis underlying nuclear size control. Intracellular pressures may derive partially from "colloid osmotic forces" from nucleic acids and other macromolecules, and thus may correlate with the degree of macromolecular crowding. As new way to measure crowding of the cytoplasm and nucleoplasm, we use micro-rheology with Genetically-Encoded Multimeric nanoparticles (GEMs) as tracer probes. Here, we tested the role of forces (and crowding?) in nuclear size control. Addition of an osmotic agent (sorbitol)

caused a decrease in both nuclear and cell volume, but NC ratio was maintained. This suggests osmotic forces in the nucleoplasm and in the cytoplasm are roughly similar. Diffusion coefficients of GEMs showed that the nucleoplasm is normally slightly less crowded than the cytoplasm. To probe the mechanism of nuclear scaling, we investigated two cases where the NC ratio is perturbed. First, we characterized the effects of compressive force on live cells using a microfluidics device in which cell proliferation in a closed container leads gradual increase in external pressure. Under compression, cells exhibited a decrease in cell and nuclear size with a significant decrease in NC ratio. Crowding was increased both in the cytoplasm and nucleoplasm, suggesting a model in which increased pressure (both mechanical and chemical) squeezes the nucleus to decrease the NC ratio. Second, we inhibited nuclear export by leptomycin B to increase the NC ratio. Initially, the nucleus expanded with increased crowding in the nucleoplasm but not cytoplasm. Subsequently, the NC ratio continued to increase as the cell grew in volume, but crowding decreased in both compartments. This case lead to a model in which nuclear size may emerge from relationships between mechanics, NC transport, biomass synthesis, membrane tension and volume growth. Together, our findings show how forces from outside the cell as well as those in the cytoplasm and nucleus contribute to nuclear size.

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Vacuole-Nuclear Envelope Organelle Contacts Promote Proper Nuclear Pore Assembly in Several Nucleoporin Mutants.

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Title: Vacuole-nuclear envelope organelle contacts promote proper nuclear pore assembly in several nucleoporin mutants **Abstract**: the nuclear envelope (NE) is composed of two lipid bilayers that separate the nucleus from the cytoplasm, and is populated with nuclear pore complexes (NPCs) that regulate transport between these compartments. NPCs are multimeric complexes of 33 nucleoporins (Nups) in yeast, five of which contain glycine-leucine-phenylalanine-glycine (GLFG) rich repeats that maintain the passive diffusion barrier and mediate active transport through NPCs. In addition to being contiguous with the ER, the NE of *Saccharomyces cerevisiae* cells physically contacts the vacuole membrane at nuclear-vacuole junctions (NVJs). NVJ regions are regulated by several proteins including Nvj1 and Mdm1, and expand when cells are starved or cellular metabolism is altered. Using live cell fluorescence and electron microscopy, we present evidence that NE-vacuole contacts significantly increase when NPC assembly and nuclear envelope structure is perturbed, and that disabling NVJ function further disruptes NPC assembly. Mutant strains that specifically lack Nup116's GLFG domain (*nup116ΔGLFG* mutants) exhibit increased contacts between the vacuole membrane and NE, particularly at semi-permissive temperatures. Interestingly, expansion of these organelle contacts coincides with increases in NE herniations and GFP-Nic96 foci formation, indicative of NPC clustering and assembly defects. Since previous studies found that nuclear import by Kap121 is disrupted in *nup116ΔGLFG* cells, we examined GFP-Nic96 localization in temperature sensitive *kap121* mutants and found that GFP-Nic96 clusters to NE foci in a similar manner. Mutant *nup120Δ* and *nup133Δ* cells, both of which impede NPC assembly and cause significant NPC clustering, also display increased levels of vacuole-NE contacts, indicating this may be a general response to perturbed NPC function and/or assembly. *MDM1* and *NVJ1* were both deleted in *nup116ΔGLFG* mutants to formally test whether NE-vacuole interactions promote proper NPC assembly; *mdm1Δ njv1Δ nup116ΔGLFG* cells display decreased NE-vacuole contacts, increased GFP-Nic96 clustering, and synthetic growth defects. Interestingly, *mdm1Δ nvj1Δ* cells also display increased amounts of GFP-Nic96 foci and mild growth defects relative to wild type cells at higher temperatures.

Thus, we reveal a novel role for NE-vacuole contacts in proper NPC assembly, principally when assembly is already partially compromised in nucleoporin mutants. Further defining how these interorganelle contacts regulate NPC assembly and NE integrity will be especially important given that NPC-mediated transport and the Mdm1 ortholog Snx1 both regulate neurological diseases.

P508/B521

Structure-function an alysis of Heh1(LEM2) and Chm7 Suggests Role for Direct-PA-binding in Nuclear Envelope Surveillance.

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The endosomal sorting complexes required for transport (ESCRT) protein Chm7 has been implicated in a nuclear envelope surveillance pathway that directly monitors the cytosolic-exposure of the integral inner nuclear membrane protein Heh1 (LEM2) in budding yeast[1-3]. To establish additional genetic tools to functionally interrogate this pathway, we have performed a systematic deletion and point-mutation analysis of both Heh1 and Chm7. We have explored how these alleles impact Chm7 nuclear envelope targeting, "activation" and functional complementation in genetic backgrounds where *CHM7* and *HEH1* are essential. In so doing, we uncovered a previously unidentified role for a short hydrophobic stretch in Chm7 that confers specific binding to phosphatidic acid (PA). An analogous sequence in human CHMP7 binds non-specifically to lipids[4]. Consistent with the idea that PA-binding might be important for Chm7 function, the use of a gain-of-function chm7 protein results in the formation of a PA-rich domain at the inner nuclear membrane. Together, our data indicate that Chm7 binds to both Heh1 and PA-rich membranes and suggests a model where nuclear envelope surveillance might require changes to local membrane composition. References: 1. Webster, B. M., Colombi, P., Jäger, J. & Lusk, C. P. Surveillance of nuclear pore complex assembly by ESCRT-III/Vps4. *Cell* **159**, 388-401 (2014). 2. Webster, B. M. *et al.* Chm7 and Heh1 collaborate to link nuclear pore complex quality control with nuclear envelope sealing. *EMBO J.* **35**, 2447-67 (2016). 3. Thaller, D. J. *et al. An* ESCRT-LEM protein surveillance system is poised to directly monitor the nuclear envelope and nuclear transport system. *Elife* **8**, (2019). 4. Olmos, Y., Perdrix-Rosell, A. & Carlton, J. G. Membrane Binding by CHMP7 Coordinates ESCRT-III-Dependent Nuclear Envelope Reformation. *Curr. Biol.* **26**, 2635-2641 (2016).

P509/B522

The Nuclear Envelope Enriched Lipin Phosphatase Ctdnep1 Links Misregulation of Lipid Metabolism to Chromosome Instability in Dividing Cancer Cells.

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Lipin1 is the key lipid metabolizing enzyme that controls ER lipid homeostasis. Lipin1 produces the precursors for triglycerides and membrane glycerophospholipids via its membrane binding domain while nuclear localized lipin1 inhibits the transcriptional activation of lipid synthesis genes. In the presence of nutrients, lipin1 is phosphorylated by mTORC1 and excluded from the nucleus. Here, we show in dividing cancer cells that CTDNEP1, a highly conserved integral membrane protein phosphatase, dephosphorylates lipin1 to counteract mTOR and in turn limit the production of glycerophospholipids in the ER and prevents chromosome instability. We demonstrate that CTDNEP1-deleted U2OS cells contain constitutively phosphorylated and reduced levels of lipin1 leading to elevated levels of

glycerophospholipids and triglycerides. In control cells, the majority of lipin1 is dephosphorylated upon inhibition of mTOR by Torin or in nutrient-poor conditions, yet lipin1 remains mostly phosphorylated in CTDNEP1-deleted cells under these conditions. Thus, CTDNEP1 dephosphorylates and stabilizes a pool of lipin by counteracting mTor in response to nutrient availability to maintain lipid homeostasis. Endogenous-GFP tagging showed that CTDNEP1 in U2 OS cells is enriched at the nuclear envelope (NE) suggesting that local regulation of lipin globally impacts ER lipid homeostasis. Increased glycerophospholipid synthesis in CTDNEP1-deleted cells causes expansion of ER membranes in mitosis leading to a faster rate of membrane wrapping around segregated chromosomes during nuclear formation and an increased incidence of micronuclei. Live imaging of CTDNEP1-deleted mitotic cells revealed ER membranes that invade the microtubule spindle region suggesting that excess ER membranes interfere with spindle assembly and chromosome segregation. Consistent with this, spindle morphology and nuclear reformation is severely disrupted after recovery from spindle microtubule destabilizing drugs. Together, these data demonstrate that NE-enriched CTDNEP1 activates a pool of lipin1 to maintain ER lipid homeostasis. The abundance of ER membranes, in turn, limit the rate of NE reformation to protect against chromosome missegregation and micronuclear formation. Thus, CTDNEP1 links mis-regulation of lipid metabolism to genome instability, two hallmarks of rapidly dividing cancer cells.

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Atg39 Drives Nuclear Envelope Remodeling and Selectively Packages Components of the Inner Nuclear Membrane for Degradation by Autophagy.

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Selective autophagy uses cargo receptors to degrade superfluous or damaged subdomains on organelles. While we have a clear conceptual understanding of how cytoplasmic organelles are cleared by autophagy, how nuclear contents can be accessed by a primarily cytosolic machinery remains enigmatic. Here, we have recapitulated likely steps in a nucleophagy pathway by taking advantage of a recently-identified membrane-spanning nuclear cargo receptor in budding yeast, Atg39. Specifically, overproduction of Atg39 is sufficient to drive the formation of nuclear blebs that form independently of other autophagy proteins. Consistent with the conclusion that these blebs are functionally relevant, we observe a remarkable sequestration of some, but not other, inner nuclear membrane (INM) proteins suggesting that elements of nuclear-cargo selectivity are preserved. Interestingly, correlative light electron microscopy and tomography shows that the nuclear blebs contain consistently-sized vesicles likely derived from the INM, enclosed by an expanded outer nuclear membrane (ONM). This nuclear envelope remodeling could occur from the outside-in as we show using a split-GFP reporter system that, even when overexpressed, Atg39 accumulates specifically at the ONM. Thus, Atg39 targeting to the ONM and the formation of nuclear blebs with INM-cargo likely occurs through a physical coupling of both nuclear membranes. Consistent with this idea, we demonstrate that the formation of the nuclear blebs depends on the presence of the Atg39 lumenal domain, which is highly suggestive of a translumenal interaction with the INM. More systematic structure-function analyses of the lumenal domain of Atg39 supports the presence of unique ONM targeting and membrane-remodeling regions, the nature of which are currently being investigated within minimal in vitro membrane reconstitution platforms. Consistent with the idea that the nuclear blebs are ultimately engaged by an autophagosome, we demonstrate their clearance by autophagy after treatment with rapamycin. Thus, we have recapitulated the clearance of INM cargo by autophagy and are in a strong position to probe the

molecular requirements for each step in this pathway while fully assessing their functional significance using more traditional methods of nucleophagy-induction.

P511/B524

Golgi Unlinking during G2 Is Essential for Correct Spindle Formation and Cytokinesis.

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The Golgi complex of mammalian cells is composed of stacks connected by tubular bridges that form a continuous membranous system, which is also known as the Golgi ribbon. The Golgi ribbon undergoes a multistep disassembly process during G2/M transition. In particular, the cleavage of the ribbon into isolated stacks during G2 is required for entry into mitosis, indicating the existence of a 'Golgi mitotic checkpoint' able to monitor the correct mitotic inheritance of the organelle. The reason for which the Golgi unlinking is required for entry into mitosis is still not known. Here we demonstrate that forcing cells to enter into mitosis with an intact Golgi ribbon causes the formation of aberrant spindles and binucleated cells. Moreover, considering the functional relationship between the Golgi and centrosome, we tested if the presence of a centrosome was required for the Golgi-dependent G2 arrest. We found that the removal of centrosomes relieves the mitotic arrest induced by an intact Golgi. We hypothesize that an intact Golgi ribbon hampers centrosome repositioning during G2 and also induces altered subcellular localization of proteins involved in spindle formation. Our current work is aimed at a detailed understanding of the mechanisms underlying the functional relationships among Golgi unlinking, centrosome repositioning, spindle development and segregation of the genetic material.

P512/B525

Vac8 Differentially Regulate the Cvt and Pmn Pathways.

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Armadillo (ARM) repeat proteins constitute a large protein family with diverse and fundamental functions in all organisms, and armadillo repeat domains share high structural similarity. However, exactly how these structurally similar proteins can mediate diverse functions remains a long-standing question. Vac8 (vacuole related 8) is a multifunctional protein that plays pivotal roles in various autophagic pathways, including piecemeal microautophagy of the nucleus (PMN) and cytoplasm-to-vacuole targeting (Cvt) pathways in the budding yeast Saccharomyces cerevisiae. Vac8 comprises an H1 helix at the N terminus, followed by 12 armadillo repeats. Herein, we report the crystal structure of Vac8 bound to Atg13, a key component of autophagic machinery. The 70-Å extended loop of Atg13 binds to the ARM domain of Vac8 in an antiparallel manner. Structural, biochemical, and in vivo experiments demonstrated that the H1 helix of Vac8 intramolecularly associates with the first ARM and regulates its self-association, which is crucial for Cvt and PMN pathways. The structure of H1 helix-deleted Vac8 complexed with Atg13 reveals that Vac8[Δ19-33]-Atg13 forms a heterotetramer and adopts an extended superhelical structure exclusively employed in the Cvt pathway. Most importantly, comparison of Vac8-Nvj1 and Vac8-Atg13 provides a molecular understanding of how a single ARM domain protein adopts different quaternary structures depending on its associated proteins to differentially regulate 2 closely related but distinct cellular pathways.

P513/B526

CDK1 Controls ESCRT-III-dependent Nuclear Envelope Regeneration during Mitotic Exit.

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Through the process of annular fusion, the Endosomal Sorting Complex Required for Transport-III (ESCRT-III) machinery has emerged as a key player in the regeneration of a sealed nuclear envelope during mitotic exit, and in the repair of this organelle during interphase rupture. ESCRT-III polymerisation at the nuclear envelope occurs transiently during mitotic exit and CHMP7, an ER-localised ESCRT-II/ESCRT-III hybrid protein, initiates this process in a manner dependent upon the INM protein LEM2. Nucleocytoplasmic transport mechanisms act to separate LEM2 and CHMP7 during interphase, however, it is unclear how CHMP7 assembly is suppressed in mitosis when NE and ER identities are mixed. Further, the integration of ESCRT-III-dependent nuclear envelope sealing with classical cell-cycle control programmes, and mechanisms to ensure correct spatio-temporal assembly of ESCRT-III during mitotic exit are currently unclear. Here, we examine phosphorylation dynamics of CHMP7 during mitotic exit and identify CDK1 phosphorylation as a regulator of ESCRT-III activity for proper nuclear envelope regeneration.

P514/B527

Helical Folding of a Hydrophobic Stretch in CHMP7 Mediates Its Targeting to Membranes with Phosphatidic Acid.

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The Endosomal Sorting Complex Required for Transport machinery is an ancient, evolutionarily conserved, multi-subunit complex that is deployed by cells to perform a diverse collection of physiological and pathophysiological processes. The membrane remodeling outcome of the ESCRT activity is mainly operated by ESCRT-III proteins which, among other functions, are involved in sealing of membrane fenestrations at several sites, including the nuclear envelope. CHMP7 is an ESCRT-II/ESCRT-III hybrid protein that transiently localizes to the resealing nuclear envelope during exit from mitosis. We have previously described an endoplasmic reticulum binding domain in CHMP7's N-terminus, in the absence of which ESCRT-III cannot subsequently assemble at the reforming nuclear envelope. However, the structural basis of membrane binding remained unclear. Here we applied co-sedimentation experiments with the recombinant protein and liposomes of different lipid composition and size to show that CHMP7's membrane binding increases upon exposure to phosphatidic acid, but it is neither affected by charged lipids nor different degrees of positive membrane curvature. Furthermore, we studied protein-lipid interactions with circular dichroism spectroscopy to reveal that interaction with phosphatidic acid causes an increase in helical content in the recombinant protein that can be traced to the hydrophobic stretch in the first winged helix domain of the ESCRT-II-like N-terminus. These data suggest that CHMP7 targeting to membranes might be dependent on the recognition of liquid disordered lipid phases in *vitro* that is reflected in the localization to areas with packing defects or negative curvature in the cell. Further structural and biophysical characterization of the membrane-bound CHMP7 will clarify the architectural details of its ER membrane targeting as well as the interplay between ESCRT-II and ESCRT-III membrane binding features.

Mitochondrial Dynamics and Morphology

P515/B528

Phosphorylation of Mitofusin GTPase Domain Regulates Fusion Activity.

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Mitochondrial structure is determined by dynamic processes including fusion, division and transport. Changes in mitochondrial structure evoke changes in mitochondrial function. To couple mitochondrial function to changes in cellular physiology, mitochondrial dynamics are regulated in response to different cellular signals. Mitochondrial fusion is mediated by large GTPases of the dynamin-related protein (DRP) family. In vertebrates, two isoforms of mitofusin, Mfn1 and Mfn2, are required for outer membrane fusion. To investigate the mechanism of mitofusin activity and its regulation in cells, we set out to identify and characterize novel post-translational modifications of Mfn1. In mass spectrometry analysis of Mfn1, we identified a modified peptide from a loop in the GTPase domain with a single conserved serine that is phosphorylated at steady state. To understand how phosphorylation might alter mitofusin activity, we have characterized the Mfn1 phosphoblocking (S-A) and phosphomimicking (S-E) variant proteins in vitro and in cells. Our data indicate that the phosphoblocking variant has wild type function while the substitution of glutamic acid at this position alters protein function. Using a minimal GTPase domain construct of Mfn1, we demonstrate that intrinsic enzyme activity is not altered by substitution of glutamic acid. Interestingly, our reconstituted mitochondrial fusion assay revealed that mitochondria with the phosphomimicking variant had severely diminished fusion activity compared to controls. To determine the basis of this impaired activity, we assessed two forms of nucleotide-dependent assembly. Consistent with diminished fusion activity, nucleotide dependent self-assembly of higher order oligomers and formation of a trans complex were both significantly impaired for the phosphomimicking variant. Therefore, this loop plays a critical role in mitofusin function. Together, our data are consistent with a model where phosphorylation of this residue decreases fusion activity by impairing mitofusin assembly.

P516/B529

Membrane Phospholipid Composition Directly Influences Mitochondrial Dynamics.

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The coordinated fusion and fission of mitochondria has been shown to be critical for maintaining the functional integrity of this essential organelle and several human diseases are associated with defective mitochondrial dynamics; including structural abnormalities that manifest during the progression of cancers, diabetes, and severe neurodegenerative disorders. Although a growing number of proteins have been identified as important regulators of mitochondrial morphology, the direct role of membrane lipids during the fusion and fission processes have not been assessed experimentally. To address these shortcomings, we devised a protein-engineering platform that would allow for the acute remodeling of defined classes of phospholipids within the outer mitochondrial membrane (OMM) of intact cells. For this, we used a modified bacterial phosphatidylinositol (PtdIns)-specific phospholipase C (bacPIPLC) scaffold to selectively hydrolyze the headgroup from PtdIns and generate a membrane-embedded diacylglycerol (DAG) backbone. Spatial restriction of bacPI-PLC activity was achieved by attaching the rapamycin-inducible FK506-binding protein (FKBP) dimerization module (FKBP-PIPLC) and co-expressing

a validated OMM-targeting sequence labeled with the FKBP-rapamycin binding domain (AKAP-FRB). Recruitment of the FKBP-PIPLC to the OMM not only caused the expected local accumulation of DAG, which we visualized using a high-affinity biosensor, but it also initiated the rapid and uniform fragmentation of the mitochondria. As important controls for these studies, targeting of a catalytically-inactive variant of the FKBP-PIPLC to the OMM failed to induce fragmentation of the mitochondria. The dramatic structural effects induced by the active FKBP-PIPLC also failed to alter the OMM potential or lumenal Ca^{2+} concentration; suggesting that the changes observed are not due to gross insults to the integrity of the OMM. Results using electron microscopy reinforce these data as well as revealed marked vesiculation of the inner mitochondrial membrane (IMM) as well as an associated loss of cristae that occurs just minutes after tethering the FKBP-PIPLC to the OMM. These phenotypic changes are reminiscent of the chronic alterations to IMM morphology that are observed in cells lacking protein machineries involved in coordinating IMM dynamics or that facilitate non-vesicular lipid transport between the OMM and IMM. Taken together, these findings highlight the unique opportunity afforded by this system to understand how rapid changes to the lipid composition of the OMM can be transmitted to the IMM as well as establish a direct relationship between lipid metabolism within the OMM and morphological changes that manifest in mitochondrial-associated diseases.

P517/B530

Ubl4A Is Critical for Mitochondrial Fusion Under Nutrition Deprivation through Arp2/3 Complex.

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Mitochondrial fusion and fission are dynamic processes regulated by the actin network. Under a nutrition starvation condition, mitochondrial fusion process is increased for energy conservation. We have previously shown that Ubl4A-deficient neonatal mice were sensitive to starvation stress during birth. The underlying mechanism is through a direct association of Ubl4A with actin-related protein Arp2/3 complex to synergize actin branching process. Here we found that mitochondria in the Ubl4A knockout newborn mouse livers were fragmented and lost integrity. Time-lapse imaging indicated that mitochondrial fusion process in the Ubl4A-deficient cells was significantly delayed under starvation. Immunostaining reveled that Ubl4A co-localized with the Arp2/3 complex on mitochondria. Interestingly, deficient in Ubl4A resulted in less Arp2/3 complex around mitochondria. Knockdown of the Arp2/3 complex in wild-type cells impaired mitochondrial fusion under nutrition deprivation. Consistently, put-back of the wild-type but not the Arp2/3-binding deficient mutant Ubl4A could rescue the starvation-induced mitochondrial elongation phenotype. These results suggest that Ubl4A-mediated Arp2/3-actin network is critical for mitochondrial fusion process under nutrition deprivation stress.

P518/B531

Understanding How Post-translational Modifications Regulate the Mitochondrial Fission Machinery in Glioblastoma.

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Mitochondria are essential and dynamic organelles that maintain a delicate balance of division and fusion in response to cellular stimuli. These processes sustain mitochondrial health and function, and alterations to either fission or fusion are associated with a variety of diseases, including cancer. Glioblastoma (GBM) is an aggressive brain cancer that takes the lives of nearly 90% of patients within 5

years of diagnosis. Glioblastoma stem cells (GSCs) represent a subset of the tumor cells that can self-renew and propagate new tumors, which contributes to higher rates of recurrence. GSCs also are resistant to conventional GBM therapies, including chemotherapy and radiation. Recent studies have observed excessive mitochondrial fission in GSCs when compared to non-stem GBM cells. This finding may represent underlying bioenergetic changes, which lead to altered post-translational modifications (PTMs) of proteins in the mitochondrial fission machinery. Dynamin-related protein 1 (Drp1), the essential mediator of mammalian mitochondrial fission, is regulated by PTMs, such as phosphorylation and O-GlcNAcylation. In addition, PTMs on Drp1's partner proteins, such as mitochondrial fission factor (Mff), may also effect the recruitment of Drp1 to the mitochondria. We propose that O-GlcNAcylation of Drp1 is an activating modification that promotes Drp1 re-localization from the cytosol to the mitochondria resulting in increased mitochondrial fission. In addition, we are examining the phosphorylation status of Mff to identify synergistic changes that favor mitochondrial division. Overall, our goal is to characterize the impact of PTMs on the fission machinery to assess functional changes leading to altered mitochondrial dynamics in GSCs. In this way, we hope to identify potential therapeutic targets that alter PTMs in GSCs to prevent recurrence in GBM patients.

P519/B532

ALS/FTD Mutant CHCHD10 and CHCHD2/CHCHD10 Loss Trigger OPA1 Processing by OPA1 and Cardiomyopathy in *Vivo*.

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Objective: Characterization of mitochondrial cristae and OPA1 processing in CHCHD2/10 DKO and mutant CHCHD10 KI mice. Introduction: Dominant mutations in the mitochondrial paralogs CHCHD2 and CHCHD10 were recently identified as causing Parkinson's disease and ALS/FTD/myopathy, respectively. Their mutation or loss has been associated with aberrant mitochondrial cristae structure in a variety of models; the mechanism, however, has been unclear. Additionally, the precise mechanism of mutant CHCHD2/CHCHD10 pathogenesis (toxic gain-of-function vs. dominant negative) and its relationship to the normal physiologic function of CHCHD2 and CHCHD10 has been unknown. Methods: analysis of cell lines and mouse heart, using transmission electron microscopy, super-resolution light microscopy, echocardiography, immunoblotting, and transcriptomics. Results: Here we report that fibroblasts from the first CHCHD2/CHCHD10 double knockout (DKO) mouse exhibit cristae abnormalities due to cleavage of the fusogenic long OPA1 isoforms by the stress-induced peptidase OMA1. OMA1 KO reciprocally altered the processing and distribution of CHCHD2/CHCHD10: intense CHCHD2/CHCHD10 foci formed in OMA1-deficient mitochondria with expanded inner membrane structures, and CHCHD2/CHCHD10 was degraded upon OMA1 activation. CHCHD10 mutation or CHCHD2/CHCHD10 knockout also caused OMA1 activation in *vivo*. Knock-in CHCHD10 S59L mice exhibited OPA1 cleavage by OMA1 coincident with development of cardiomyopathy and activation of the mitochondrial integrated stress response (mt-iSR); cardiomyopathy with OMA1 and mt-iSR activation were partially phenocopied in CHCHD2/CHCHD10 DKO mice. Summary: OPA1 cleavage by activated OMA1 is identified as a common response to mutant CHCHD10 and CHCHD2/CHCHD10 double knockout in cell culture and in *vivo* and is at least partially responsible for observed cristae abnormalities in these models. mt-iSR and development of cardiomyopathy are additionally phenocopied in CHCHD2/CHCHD10 DKO and mutant CHCHD10 mice. Conclusion: These findings link the physiologic role of CHCHD2/CHCHD10 in maintaining L-OPA1 stability to the pathogenesis of mutant CHCHD10. Additionally, they identify OMA1 as a

potential therapeutic target in CHCHD2/CHCHD10-related disease. Acknowledgement: This research was supported (in part) by the Intramural Research Program of the NIH, NINDS

P520/B533

MCL-1 Is an Essential Modulator of Mitochondrial Dynamics in Stem Cells and Cardiomyocytes.

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MCL-1 (myeloid cell leukemia-1) has been well-characterized as an anti-apoptotic protein. Recent studies from our laboratory also implicate MCL-1 as a novel modulator of mitochondrial dynamics and metabolism at both the outer mitochondrial membrane and the matrix. My studies show that MCL-1 downregulation in stem cells induces dramatic changes to the mitochondrial network, as well as the loss of the key pluripotency transcription factors, NANOG and OCT4. Mechanistically, this change in mitochondrial network morphology is due, at least in part, to MCL-1 interaction with DRP-1 (dynamin-related protein 1) and OPA1 (optic atrophy 1), two GTPases responsible for regulating mitochondrial dynamics. Depletion of MCL-1 affected the rates of mitochondrial fission and caused a shift in the metabolic profile of stem cells, pointing to MCL-1 as a potential mediator of mitochondrial dynamics and metabolism. To decipher the molecular mechanism for MCL-1 regulation of DRP-1 and OPA1, we are currently performing structure-function analyses coupled with high resolution imaging to examine the effects of disrupting these protein interactions. We hypothesize that MCL-1 is essential for mitochondrial homeostasis by modulating mitochondrial morphology and function, independently of its canonical role in apoptosis. Recently discovered small molecule inhibitors of MCL-1 are being tested as chemotherapeutic agents for MCL-1-sensitive tumors. Our preliminary studies point to a conserved role of MCL-1 in maintaining mitochondrial integrity in human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). Inhibition of MCL-1 in iPSC-CMs has significant effects on mitochondrial morphology and myofibril assembly, even in the absence of caspase activation. Functionally, MCL-1 inhibition caused significant defects in cardiomyocyte contractility and beating rates. Elucidating these alternative functions of MCL-1 in human cells will be crucial to reveal potential adverse effects caused by MCL-1 inhibitors currently being tested in the clinic. Our studies aim to uncover the mechanisms by which MCL-1 and the BCL-2 family of proteins control mitochondrial dynamics and metabolism during reprogramming and differentiation.

P521/B534

α-synuclein Has Distinct in *Vivo* Roles in Mitochondrial Dynamics and Mitochondrial Homeostasis.

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The balance between fission via dynamin-related protein (DRP1) and fusion via mitofusin (MFN), which occur in concert to mitochondrial motility, is critical for mitochondrial health in axons. Defects in fission-fusion and disruption of mitochondrial transport within axons have been linked to mitochondrial dysfunction and neurodegeneration. While α-synuclein (α-syn) has been proposed to influence mitochondrial dynamics within axons, the mechanistic details of how α-syn effects mitochondrial homeostasis remains unknown. We found that excess α-syn causes mitochondrial fragmentation, similar to excess DRP1, but in contrast to excess MFN2, which caused elongated mitochondria. Intriguingly, excess or depletion of DRP1 rescued α-syn-mediated mitochondrial fragmentation, while excess MFN

had no effect. Deletion of the acidic C-terminal domain of α-syn (α-syn1-120) which is thought to hinder membrane binding, or the NAC domain (α-synΔNAC) which is thought to promote self-aggregation did not affect α-syn-mediated mitochondrial fragmentation. Further, α-syn-mediated mitochondrial fragments were oxidized as assayed by the in *vivo* oxidation marker MitoTimer. However, α-syn 1-120-mediated fragmented mitochondria were healthier. Intriguingly, while α-syn-mediated oxidized mitochondrial fragments showed biased retrograde motility, α-syn 1-120-mediated healthy mitochondrial fragments did not. Together our observations suggest that mitochondrial size is independent of mitochondrial health in *vivo*, and demonstrates that the N and C-terminal regions of α-syn have novel in *vivo* roles during mitochondrial fission and oxidation. Therefore, our findings provide a novel physiological role for α-syn during mitochondrial homeostasis, and highlight a common pathological mechanism for targeted therapeutics early before neuronal loss or clinical manifestation of Parkinson's disease.

P522/B535

Pink1 Promotes Mitochondrial Fission and Its Essential for Porcine Embryonic Development.

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Phosphatase and tensin homolog (PTEN)-induced kinase 1 (PINK1) selectively locates to the outer membrane of impaired mitochondria and promotes their autophagy. On the other hand, PINK1 regulates mitochondrial morphology by interacting with mitochondrial fission and fusion components, shift mitochondrial fusion/fission balance towards more fission. In mammalian, the oocytes and early embryos has approximately 2×10^5 mitochondria, at least one or two orders of magnitude more than somatic cells, but few studies focus on the mitochondrial dynamics during preimplantation embryo development. To investigate whether PINK1 are required for mitochondria dynamics in porcine preimplantation embryos, gene knockdown and inhibitors were engaged in the present study, and mitochondrial dynamics were observed by transmission electron microscope. The results showed that knockdown of PINK1 significantly impaired blastocyst formation and quality. In addition, TEM analysis revealed that knockdown of PINK1 induced mitochondrial elongation and swollen. Mitochondrial DNA (mtDNA) copy number was reduced after PINK1 knockdown. Elongated mitochondrial induced by PINK1 knockdown further caused mitochondrial dysfunction, oxidative stress and ATP deficiency. Finally, both of the autophagy and apoptosis were significantly increase after mitochondrial dysfunction. However, over expression of Drp1 prevented mitochondrial elongation and embryo development impairments induced by PINK1 knockdown. In conclusion, these data suggest that PINK1 promotes mitochondrial fission in porcine preimplantation embryos.

P523/B536

Mtfr2 Is Required for Mitochondrial Fission and Accurate Chromosome Segregation.

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Mitochondria change shapes through the combined action of fusion and fission. The crosstalk between mitochondria dynamics and cell division has drawn lots of attention in recent years. In this study, we characterized MTFR2 and explored its role in mitochondrial fission and cell division.MTFR2 is located on the outer membrane of mitochondria by both super-resolution and laser-scanning confocal microscopy Domain-mapping showed the first 50 amino acids of MTFR2 was sufficient for its mitochondrial

targeting. Overexpressing MTFR2 in Hela cells promoted DRP1 dependent mitochondrial hyper-fission even in G1/S arrested cells. DRP1 is a dynamin-related large GTPase whose polymerization is essential for mitochondrial fission. On the other hand, depleting MTFR2 through RNAi or CRISPR-Cas9 resulted in longer and interconnected mitochondrial. Though the expression level, as well as the DRP1 localization, were not altered after MTFR2 depletion, immunoprecipitation showed MTFR2 depletion affected the polymerization of DRP1, but MTFR2 and DRP1 had no direct interaction.MTFR2 is the only known mitochondrial fusion/fission factor that is co-transcribed with core centromere/kinetochore components. MTFR2 is phosphorylated during mitosis, similar to the canonical mitochondrial fission regulating GTPase DRP1. Time-lapse live-cell imaging demonstrated MTFR2 depletion extended the average duration from nuclear envelope breakdown to anaphase onset (59min vs 141min). Expressing wild type MTFR2 but not MTFR2 mutants found in cancer samples in the knockout cells rescued the defect, suggesting MTFR2 is important for normal cell division. Interestingly, it was also found that chromosomal condensation was compromised in MTFR2 knockout cells.In conclusion, MTFR2 functions to regulate proper mitochondrial fission and chromosomal segregation. More detailed mechanistic studies on MTFR2 will contribute to further understanding of the function of mitochondria and mitochondrial proteins in cell division.

P524/B537

Using Single-Molecule Localization Microscopy to Study Protein Organization and Function at a Molecular Level to Gain a Better Understanding of Many Biological Phenomena.

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Studying protein organization and function at molecular level is a major technical challenge that limits our understanding from both a diagnostic and treatment perspective. Single-molecule localization microscopy (SMLM) is a Nobel Prize winning technique that allows quantitative imaging below the diffraction limit and can be applied to significantly improve our understanding of many biological phenomena. ONI has created the world's first desktop super-resolution microscope, the Nanoimager, for imaging single molecules. The Nanoimager is a compact and state-of-the-art microscope, offering quantitative analysis for localization-based imaging (dSTORM and PALM), single-particle tracking and single-molecule FRET. With the addition of SIM/confocal imaging capabilities, the Nanoimager provides a versatile imaging platform with unrivalled stability and flexibility to work on a lab bench.The data presented was acquired using two-color, super-resolution imaging of the mitochondrial markers, TOM20 and HSP60, to clearly resolve the sub-mitochondrial compartment and reveal their spatial organization at 20 nm resolution. Moreover, optical sectioning using Structured Illumination Microscopy (SIM) in three colors showed the co-localization of mtDNA-associated proteins along the mitochondrial network. Finally, live-cell time-lapse imaging and automated particle tracking analysis was used to investigate Drp1 dynamics and interactions with the mitochondria. This presentation provides evidence of the use of SMLM imaging as a powerful tool for rapid and multiplexed mitochondria characterization in combination with unique structural insight. They have demonstrated the wide-ranging applicability of the Nanoimager in different biological applications encompassing the field of immunology, neurobiology, cell biology and cancer.

P525/B538

Opa-1 Deficiency Promotes Muscle Atrophy through Upregulating Er-Mito Contacts and Autophagy.

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Type 2 Diabetes (T2DM) in some patients has been shown to have dysregulated Mitochondria Associated Membranes (MAMs) and decreased muscle mass. MAMs are enriched with specific proteins and lipids that aid in specialized structural rearrangements such as the formation of autophagosomes. Decreased optic atrophy protein-1 (OPA-1) in skeletal muscle has been reported in both murine and human T2DM models. Loss of OPA-1 in skeletal muscle has been observed to cause muscle atrophy and increase mitochondria associated membrane-tethering protein Mitofusin-2 (MFN-2). The Framingham Offspring Study has shown that high plasma branch amino acids (BCAAs) concentrations were linked to an increased risk of T2DM and BCAAs have been observed to regulate autophagosome recruitment through interactions with mammalian target of rapamycin complex (mTORC1). Therefore, we hypothesized that OPA-1 deficiency induces catabolism of skeletal muscle resulting in an increase in BCAAs that recruit autophagosomes to the MAM space (MAI). To investigate the role of OPA-1 on MAI, studies were performed in OPA-1 deficient mice and OPA-1 ablated skeletal muscle cells. The ablation of OPA-1 in skeletal muscle showed a decrease in soleus, gastrocnemius, quadriceps, and tibialis anterior muscle weight. Notably, loss of OPA-1 in gastrocnemius muscle showed an increase in the following MAM proteins that are important for MAI tethering which include: MFN-1, MFN-2, and PACS2. Loss of OPA-1 also increased the MAM autophagosomal recruiting protein, ATG5. qPCR analysis after the deletion of OPA-1 in 40-week skeletal muscle displayed an increase in MFN-1, MFN-2, LC3B, ATG7, and ATG5 transcripts, while p62 transcripts were shown to decrease. Primary myoblast and myotubes were generated by isolating satellite cells from OPA-1 floxed mice, differentiated, and subsequently the deletion of OPA1 by infecting the cells with adenoviral Cre recombinase. Ablation of OPA-1 in myotubes increased the following MERC proteins: MFN-1, MFN-2, and PACS2. TEM an alysis of OPA-1 deficient skeletal muscle and myotubes had an increase in MAI and lysosome structures. An alysis of amino acid metabolite peak intensities heat map indicated that an increase in Isoleucine, Leucine, and Valine occurred after the deletion of OPA-1 in skeletal muscle. This data suggests that loss of OPA-1 increased MAIs, which may lead to decreased muscle mass and that amino acids may contribute to the recruitment and formation of autophagosomes.

P526/B539

Opa-1 Deficiency in Skeletal Muscle Increases Mitochondria-er Contact Formation through an Atf-4 Dependent Mechanism.

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Defective mitochondria-associated membranes (MAMs) or mitochondria and endoplasmic reticulum (ER) contact sites (MERCs), has been associated with insulin Resistant Type 2 Diabetes Mellitus (T2D). MERCs are enriched with specific proteins and lipids that are believed to mediate inter-organellar communication such as calcium and lipid transfer, autophagosome formation, regulation of mitochondrial morphology, and apoptosis. Previously, we demonstrated that OPA-1 deficiency in skeletal muscle induces ER stress, which correlated with upregulation of Mitofusin-2 (MFN-2), which is a

known tethering protein in MERCs. Therefore, we hypothesized that OPA1-induced mitochondrial stress results in narrowing of MERC distance through the upregulation of tethering proteins. To test this hypothesis, we surveyed for several MERCs proteins in OPA1-deficient skeletal muscle samples and primary myoblasts. Primary myoblasts were generated by isolating satellite cells from OPA-1 floxed mice and subsequently deleting OPA1 by infecting the cells with adenoviral Cre recombinase. Ablation of OPA-1 in myoblasts increased the following MERC proteins: MFN-1, MFN-2, BIP. Similarly, loss of OPA-1 in skeletal muscle increased MFN-1, MFN-2, PACS-2, GRP75, BIP, and IP3R. qPCR was done to assess gene expression, ER stress genes (ATF4, ATF6, BIP, CHOP) and MERC genes (IP3R3, GRP75, VDAC3, MFN-2) increased after the deletion of OPA-1 in myoblasts and skeletal muscle. An alysis of proximity ligation assay revealed that MFN-1 and MFN-2 interactions are increased in OPA-1 deficient cells and that VDAC and IP3R and MFN-1 and MFN-2 interactions were increased in OPA-1 deficient skeletal muscle. TEM an alysis of OPA-1 deficient myoblasts and soleus skeletal muscle confirmed an increase in the number of MERCs and a reduced distance between ER and Mitochondria. TUDAC treatment decreased ER stress Gene Expression (ATF4 and CHOP) and MERC gene expression (IP3R3, GRP75, VDAC3, MFN-2) in OPA-1 deficient skeletal muscle compared to control. Promoter analysis of a CHIPseq data set revealed that ATF4 and CHOP bind to the promoters of MERCs that are upregulated in OPA-1 deficient samples. Collectively, these data suggest that loss of OPA-1 in muscle cells results in the induction of MERC proteins, which correlates with increased MERCS formation. Increased MERCs may represent a compensatory mechanism in response to mitochondrial stress that is precipitated by OPA1 deficiency.

P527/B540

Investigating the Mechanism and Regulation of Mitochondria-dependent Dynein an choring.

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The distribution of organelles is closely linked to their function and inheritance in cells. One protein in budding yeast, Num1, serves to position and aid in the inheritance of both mitochondria and the nucleus. Specifically, Num1 anchors mitochondria as well as dynein to the cell cortex, and this anchoring activity is required for proper mitochondrial distribution and dynein-mediated nuclear inheritance. We have previously shown that mitochondria drive the assembly of Num1 clusters, which then serve as cortical attachment sites for dynein. Using a synthetic clustering system to bypass the requirement of mitochondria for Num1 cluster formation, we were able to create two populations of Num1 clusters, mitochondria-associated and non-associated clusters. We found that dynein was preferentially anchored to mitochondria-associated clusters. We are now using a structure-function approach to dissect the mechanism and regulation of this mitochondria-dependent dynein anchoring. Using a series of Num1 truncations in our synthetic clustering system, we have identified a region of Num1 that is important for the mitochondria-dependent association of dynein with Num1. In the absence of this region, we find a significant increase in cortical dynein anchoring at Num1 clusters that are not associated with mitochondria. These data suggest that this region of Num1 negatively regulates cortical dynein anchoring. Consistently, we find that in our synthetically clustered system, cells lacking this region of Num1 exhibit increased dynein activity. Based on these data, we hypothesize that this identified region of Num1 promotes an arrangement of the protein within a cluster that favors dynein anchoring in the presence of mitochondria and, consequently ensures dynein anchoring occurs after mitochondrial inheritance. The spatiotemporal integration of mitochondrial and nuclear positioning pathways likely contributes to the regulated order of organelle inheritance during the cell cycle.

P528/B541

Regulated Expression of the Autophagy Receptor NIX Initiates Mitochondrial Fission Via DRP1 to Drive Proper Epidermal Differentiation.

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Epidermal keratinocytes degrade all intracellular organelles during a specialized process of terminal differentiation to form compact cornified layers that provide a protective outer barrier for the body. To understand how keratinocytes initiate this process of programmed organelle break-down, we coupled live confocal microscopy with organotypic human epidermis to visualize single-organelle dynamics in the stratified differentiated tissue. In the uppermost epidermal layers, we found that mitochondria transform from interconnected tubules to spherical fragments, suggesting an up-regulation of fission. Coincident with this morphological change, upper layer keratinocytes in both organotypic cultures and in normal human skin accumulate high levels of NIX, a membrane-tethered mitophagy receptor known to be induced by hypoxia. We hypothesized that increased levels of NIX could initiate mitochondrial fission and mitophagy during terminal keratinocyte differentiation. In agreement with this, ectopic expression of NIX in undifferentiated keratinocytes was sufficient to induce mitochondrial fragmentation and drove premature differentiation of organotypic epidermis. Toward a mechanism, we found that NIX enhanced mitochondrial localization of FIS1, a receptor for the membrane fission GTPase dynamin-related protein 1 (DRP1). Accordingly, NIX also enhanced recruitment of DRP1 to the mitochondrial outer membrane and increased DRP1 levels in mitochondrial fractions of cell lysates. Moreover, using a dominant-negative DRP1 (K38A), we confirmed that NIX required DRP1 activity to induce mitochondrial fragmentation. Finally, we found that expression of DRP1-K38A or treatment with the DRP1 inhibitor Mdivi-1 impaired mitochondrial fragmentation and resulted in aberrant morphogenesis of organotypic epidermis, indicating DRP1-dependent mitochondrial fission is essential for epidermal tissue differentiation. Based on our findings, we propose a model in which NIX expression is up-regulated in the outer layers of the epidermis to properly initiate mitochondrial fragmentation via DRP1 to initiate terminal differentiation and epidermal barrier formation.

P529/B542

Two Forms of Opa1 Coordinate to Induce Mitochondrial Inner Membrane Fusion.

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Mitochondrial membrane dynamics is a cellular rheostat that relates organelle morphology and metabolic function. Using an in *vitro* reconstitution system, we describe a mechanism for how mitochondrial inner-membrane fusion is regulated by the ratio of two forms of Opa1. We found that the long-form of Opa1 (l-Opa1) is sufficient for membrane docking, hemifusion and basal levels of content release. However, stoichiometric levels of the processed, short form of Opa1 (s-Opa1) work together with l-Opa1 to mediate efficient and fast membrane pore opening. Additionally, we found that excess levels of s-Opa1 inhibit fusion activity, as seen in conditions of abnormal cellular proteostasis. These observations describe a tunable mechanism for gating membrane fusion.

P530/B543

Spatial Organization of Mitochondrial Inner Membrane Subdomains.

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The mitochondrial inner membrane is an essential platform to perform and integrate key cellular pathways ranging from oxidative phosphorylation to mitochondrial genome replication and expression. As such, the inner membrane is protein-dense and highly organized into at least three morphologically and compositionally distinct domains: cristae, cristae junctions, and boundary regions. Based on an analysis of a small fraction of the inner membrane proteome, it is thought that the electron transport and respiratory complex assembly machinery are distinctively localized to cristae while in contrast, solute transporters and protein import machinery are enriched in boundary regions. To gain insight into the mechanisms underlying the domain sorting of inner membrane proteins, we systematically determined the subdomain localization of a majority of inner membrane-associated proteins in budding yeast. We resolved the domain localization of fluorescently-tagged versions of inner membrane proteins by light microscopy by expanding the diameter of mitochondria in vivo using a conditional mutation of MDM12, which encodes an essential component of the ER-mitochondria encounter structure. Our results indicate that a majority of GFP-tagged inner membrane proteins localized to cristae, as opposed to boundary regions, and a majority of respiratory and protein import machinery localized to cristae and boundary regions, respectively. However, there were a number of exceptions including a cristae-localized solute transporter, Yea2, and boundary-localized respiratory assembly protein, Rcf2. Our data suggest a simple model in which boundary and cristae regions are generated by retention of a majority of proteins in cristae.

P531/B544

3D-SIM/FIB-SEM Allows Correlation of Mitochondrial Membrane Potential and Nucleoid Distribution with Mitochondrial Ultrastructure.

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Mitochondria are double-membrane organelles that carry out numerous cellular functions including oxidative phosphorylation, calcium buffering, and lipid synthesis. Mammalian cells can contain hundreds or even thousands of mitochondria that organize into a dynamic pleiomorphic network. Within this network, individual mitochondria are both structurally and functionally diverse. Understanding and better characterizing mitochondria heterogeneity within a single cell is a major ongoing effort that has been hampered by the lack of methods available to correlate mitochondria functional state with their morphology and ultrastructure. Here we describe a pipeline that enables the correlation of mitochondrial membrane potential obtained by cryogenic superresolution microscopy with ultrastructural information obtained by 3D electron microscopy (EM). Specifically, we plated U2OS cells on sapphire coverslips and incubated cells with TMRM, a cationic dye that can be used to estimate mitochondrial membrane potential, and SybrGreen, a DNA dye that can selectively label mtDNA nucleoids. The cells were then high pressure frozen and imaged by cryogenic 3D-Structured Illumination Microscopy (SIM). Whereas TMRM and SybrGreen are typically lost from mitochondria upon conventional chemical fixation, we found that both dyes retained their mitochondrial localization in the

vitreously frozen cells. Next, cells were processed for EM by freeze substitution, heavy metal staining, and embedding in Eponate 12 resin. Finally, cells were imaged by Focused Ion Beam Scanning EM (FIB-SEM) with isotropic 8 nm voxels and the resulting EM stack was manually aligned with the 3D-SIM data using the BigWarp ImageJ plugin. In the aligned data, we observed that TMRM robustly correlated with the position of mitochondria cristae, while SybrGreen-marked nucleoids appeared to instead localize to regions with decreased cristae density. Moving forward, we plan to use this correlative approach to explore how mitochondrial membrane potential varies both across and within mitochondria, whether membrane potential is locally influenced by mitochondria-organelle contacts, and finally how mtDNA nucleoid number scales to mitochondria volume.

P532/B545

Patient Mutation in CHCHD10 Causes Myopathy and Oma1-dependent Mitochondrial Fragmentation.

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The paralogous mitochondrial proteins CHCHD2 and CHCHD10 have been recently identified as autosomal dominant causes of Parkinson's disease and ALS/FTD (as well as myopathy), respectively, when mutated. Interestingly, these mutations also lead to mitochondrial DNA (mtDNA) instability. The function of these proteins, as well as the mechanism by which their mutations lead to disease, remain unknown. The currently prevailing model of pathogenicity involves mutations in the proteins leading to protein aggregation and maladaptive activation of the mitochondrial integrated stress response, ultimately leading to cell death (the toxic gain-of-function model). However, recent work by our lab shows that the same mitochondrial stress response pathway is activated by knocking out both CHCHD2 and CHCHD10. Additionally, the identification of a new pathogenic mutation (G58R) whereby a hydrophobic amino acid is substituted by a hydrophilic one (thus decreasing propensity to aggregate) also does not fit within the aggregation model. Recent work by our lab also identified cleavage (and inactivation) of the mitochondrial shaping protein OPA1 by the peptidase OMA1 in CHCHD10 KI and CHCHD2/CHCHD10 KO models. We have developed a KI mouse model of the G58R mutation, which, in humans, causes a cardiomyopathy. We show that the G58R mouse recapitulates the cardiomyopathy phenotype more profoundly than the previously described S59L KI model. Muscle wasting is evident from the decreased weights of the G58R mouse compared to WT. Behaviorally, the G58R mouse displays significant motor and balance impairment when assessed for grip strength, rotarod balance and time until fatigue on a treadmill. We see OMA1 activation and cleavage of OPA1 in tissue obtained from the G58R mouse. Additionally, we will assess for CHCHD10 aggregation and analyze mtDNA for deletions and copy number depletion. In summary, we describe in this study a new mouse model for myopathy that provides insights into alternative mechanisms underlying the pathogenesis of CHCHD2/CHCHD10 mutations.

Mitochondrial Contacts with Other Organelles

P533/B546

Human Beta-glucosidase 2 (GBA2) Contains a Functional Mitochondrial Targeting Domain.

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Beta-glucosidase 2 (GBA2) is an enzyme involved in the metabolism of sphingolipids, it cleaves glucosylceramide into glucose and ceramide. GBA2 is highly sensitive to inhibition by the clinically licenced drug miglustat (Zavesca®), which passes the blood-brain barrier but does not cause major adverse effects. The physiological significance of the GBA2 enzymatic activity is therefore not clear. By contrast, mutations in the human *GBA2* gene are associated with hereditary spastic paraplegia 46 (SGP46) and Marinesco-Sjögren-like syndrome (MSS), which are neurological diseases on the ataxia-spasticity spectrum. To establish why pharmacologically inhibiting of GBA2 does not, but mutations in the *GBA2* gene do result in neurological diseases, we characterized a number of GBA2 mutants. We compared the cellular location of wild-type GBA2 (WT, 927 amino acids) with those of GBA2 mutants. While previous studies have suggested GBA2-WT to be localized at the ER, Golgi, and plasma membrane, we detected GBA2-WT at the plasma membrane, and, in a minority of cells, in the mitochondrial matrix, using confocal and electron microscopy. GBA2 mutants carrying single amino acid-substitutions were located at the plasma membrane, as judged by confocal microscopy. In contrast, confocal and electron microscopy showed that C-terminally truncated forms of GBA2 terminating after residues 233 or 339 were localized in the mitochondrial matrix. In addition, these mutants caused mitochondrial fragmentation and loss of membrane potential. By deletional analysis, we found that amino acids 160-200 were essential for the mitochondrial localization of the GBA2-339 mutant. Our results were supported by in *silico* analysis of the GBA2-339 amino acid sequence, identifying an internal mitochondrial targeting domain centered around amino acids 161-176. Altogether, we have established that GBA2 encompasses a functional mitochondrial targeting domain, which may be responsible for mitochondrial import of GBA2 under particular conditions. These findings raise the possibility of a physiologically relevant role for GBA2 in mitochondria, separate from its enzymatic activity. Further, in individuals with mutations in the *GBA2* gene, aberrant or defective mitochondrial localization of various mutant forms of GBA2 may cause neuronal dysfunction. Our work therefore provides new insights in the cell biology of GBA2 and the cell biological basis of neurological diseases. This work was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada, Dalhousie University Faculty of Medicine, Dalhousie University Department of Pediatrics, the IWK Health Centre (Halifax, Nova Scotia), and the Nova Scotia Health Research Foundation (NSHRF).

P534/B547

Gem1/Miro1 Promotes Mitochondrial-Derived Compartment Formation at ER-Mitochondria Contact Sites.

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During cellular stress, mitochondria initiate various quality control mechanisms to promote proper mitochondrial function. Recently, we discovered a novel mitochondrial quality control system that is conserved from yeast to mammals, called the mitochondrial-derived compartment (MDC) pathway. Our current data suggests that this system protects mitochondria from nutrient stress by selectively

removing metabolite transporters from mitochondria in response to elevated nutrients. Selective transporter removal occurs by sorting into a specialized membrane-bound compartment formed from mitochondria in conditions of nutrient excess, called an MDC. Although we know mitochondrial metabolite transporters are targeted by this pathway, we understand little about how cells form MDCs. To address this question, we performed super-resolution time-lapse imaging and microscopy-based screens in yeast to elucidate the machinery and mechanism of MDC formation. Through these approaches, we discovered that MDCs are large, organelle-like structures that localize to sites of ER-mitochondria contact. MDC formation requires the ERMES (ER-mitochondria encounter structure) complex and the ERMES-associated GTPase Gem1. Mutational analysis of Gem1 demonstrated that Gem1's GTPase domains, but not EF-hands, are required for MDC formation. Similar to our observations in yeast, we found that mouse embryonic fibroblasts (MEFs) lacking Miro1, the mammalian ortholog of Gem1, fail to form MDCs efficiently and that MDCs associate with Miro1 and ER tubules in MEFs. The specific role of ER-mitochondria contact sites in formation of yeast and mammalian MDCs is currently unclear. However, our preliminary data suggests that components of ER-mitochondria contacts sites may interact with cytoskeletal elements to promote formation of these specialized mitochondrial structures. Together, these results begin to illuminate the mechanisms underlying formation of MDCs, and identify a conserved function of the Gem1/Miro1 GTPase in nutrient-dependent mitochondrial remodeling.

P535/B548

Dissecting the Physiopathological Role of Sigma-1 Receptor Mutationin Distal Hereditary Motor Neuropathies.

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Distal Hereditary Motor Neuropathies (dHMNs) are clinically and genetically heterogeneous neurological disorders characterized by degeneration of the lower motor neurons. To date, more then 20 dHMN genes have been identified but the genetic causes of most of the dHMN cases remain unknown. We have recently identified two causative mutations (E138Q and E150K) in the sigma-1 receptor (sigma-1R) encoded by the *SIGMAR1*gene, found in two Italian families affected by an autosomal recessive form of dHMN.Sigma-1R is a highly conserved chaperone protein of the endoplasmic reticulum (ER) which shares no homology with any mammalian protein. We found that sigma-1R substitutions behave as "loss-of-function" mutations affecting cell viability and altering Ca^{2+} homeostasis due to the derangement of ER-mitochondria tethering in neuroblastoma cells.Currently, we focused on the dissection of the molecular mechanisms underlying sigma-1R role in the etiopathology of dHMN using human skin fibroblasts from patients homozygous for the E150K mutation. We analyzed intracellular sigma-1R protein distribution and organelle morphology, and we investigated the global intracellular Ca^{2+} dynamics and the extent and distribution of ER-mitochondria tethers, which are one of the main determinants of mitochondria Ca^{2+} signalling. Our data clearly point out a significant disorganization of ER-mitochondria contact sites, and a consequent impairment of the global Ca^{2+} handling in patient fibroblasts compared to controls. Then, our electron microscopy analysis reveals a significant alteration of mitochondrial cristae in mutant fibroblasts compared to controls. Furthermore, patient cells show autophagy disturbance and upregulation of the Unfolded Protein Response (UPR) pathway. Concluding,

our data demonstrate the involvement of sigma-1R in the maintenance of cell homeostasis and protein quality control, highlighting its crucial role in the establishment of organelle structure, global Ca^{2+} signalling and proteostasis. Our data suggest that SIGMAR1gene mutations can cause motor neuron dysfunction in dHMN and point to the dysregulation of sigma-1R function as a critical aspect of neuronal degeneration in human neuropathies.

P536/B549

Decreased Pdzd8-mediated ER-mitochondria Contacts in Neurons Improve Fitness by Increasing Mitophagy.

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Organelle contacts are emerging as a critical biological interface playing many important roles ranging from regulation of calcium dynamics, mitochondrial fission and lipid biogenesis. Among the many roles of ER-mitochondrial contact sites is to help mediate mitochondrial quality control. The complex architecture combined with the longevity of neurons makes maintaining healthy neuronal mitochondria particularly important and challenging. We find that reducing ER-mitochondrial contacts by neuron-specific knockdown of *Pdzd8*, a recently identified tethering protein (Hirabayashi, Kwon et al., Science 2017) prolongs the locomotor activity and lifespan of *Drosophila melanogaster.* This phenotype correlates with an increase in mitophagy, suggesting that increased mitochondrial turn over and therefore improved mitochondrial quality control helps support neuronal health with aging. In contrast increasing ER-mitochondria contacts by expression of a synthetic tethering construct in neurons reduces the lifespan and general activity of the flies by disrupting mitochondrial transport and synapse formation. We also show that reducing ER-mitochondria contacts can rescue the motility defects in a fly model over-producing Amyloid-β and prolong the survival of flies fed with mitochondrial toxins. Together this data provides the first in vivo evidence that ER-mitochondria contacts mediated by the tethering protein PDZD8 are involved in mitochondrial maintenance and play a critical role in neuronal function and organismal fitness.

P537/B550

Mitochondria-Lysosome Contact Site Regulation of Mitochondrial Dynamics and Dysfunction in Charcot-Marie-Tooth Type 2.

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Both mitochondria and lysosomes are essential for maintaining cellular homeostasis, and dysfunction of both organelles has been observed in multiple neurodegenerative diseases. We recently found that mitochondria-lysosome contact sites formed dynamically and were distinct from damaged mitochondria targeted into lysosomes for degradation. Contact formation was promoted by active GTP-bound lysosomal RAB7, and contact untethering was mediated by recruitment of the RAB7 GTPase-activating protein TBC1D15 to mitochondria by FIS1 to drive RAB7 GTP hydrolysis and thereby release contacts. Functionally, lysosomal contacts marked sites of mitochondrial fission, allowing regulation of mitochondrial networks by lysosomes, whereas conversely, mitochondrial contacts regulated lysosomal RAB7 GTP hydrolysis via TBC1D15. In addition, mitochondria undergo fission and fusion events, but the dynamics and regulation of a third event of inter-mitochondrial contact formation remain unclear. Using

super-resolution imaging, we demonstrated that inter-mitochondrial contacts frequently formed and played a fundamental role in mitochondrial networks by restricting mitochondrial motility. Inter-mitochondrial contact untethering events were marked and regulated by mitochondria-lysosome contacts which were modulated by RAB7 GTP hydrolysis. Moreover, inter-mitochondrial contact formation and untethering were further regulated by Mfn1/2 and Drp1 GTP hydrolysis respectively. Surprisingly, endoplasmic reticulum tubules were also present at inter-mitochondrial contact untethering events, in addition to mitochondrial fission and fusion events. Importantly, we found that multiple disease mutations linked to the peripheral neuropathy Charcot-Marie-Tooth Type 2 such as Mfn2 (CMT2A), RAB7 (CMT2B) and TRPV4 (CMT2C) converged on prolonged inter-mitochondrial contacts and defective mitochondrial motility, highlighting a role for mitochondria-lysosome contact regulation of inter-mitochondrial contacts in mitochondrial network regulation and neurodegeneration.

P538/B551

Dissecting the Crosstalk between Lysosomes and Mitochondria.

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Mitochondria perform a wide variety of important cellular functions, ranging from energy production, to metabolism, to innate immune signaling. Mitochondria do not exist in isolation, but in a wider cellular context in which their structure and function depends on the presence of other cellular organelles. Lysosomes, in particular, heavily influence mitochondrial function, and the lysosome-mitochondrial relationship is important for maintaining mitochondrial health. Disruption of this organelle interdependence is thought to be at the heart of cellular aging and cause numerous age-related diseases, but what drives the functional connection between lysosomes and mitochondria remains unknown. We carried out genetic screens in yeast to uncouple lysosomes (vacuoles in yeast) from mitochondria and better understand the mechanics of the vacuole-mitochondria relationship. From these screens, we found that an increase in cellular iron uptake restored mitochondrial respiration in the absence of vacuolar function. Likewise, iron suppressed defects in mitochondrial structure and function brought on by vacuolar dysfunction. These data suggest that the main role of vacuoles in mitochondrial function is maintenance of a pool of bioavailable iron. Consistent with this idea, vacuole-deficient cells are starved for iron, despite being iron replete. The lack of iron bioavailability caused by vacuole impairment was exacerbated by amino acids, in particular, cysteine. Given that an important role of the vacuole is the compartmentation of amino acids, and failure of vacuoles would result in a buildup of cytoplasmic amino acids, we propose that excess cytoplasmic amino acids cause a lack of iron bioavailability which negatively impacts mitochondrial function. How cytoplasmic cysteine limits iron bioavailability is an open question, but our data suggest a role for reactive oxygen species, known disruptors of iron homeostasis, in the process. Together, this work sheds light on the vacuole-mitochondria connection, and establishes compartmentation of amino acids as an important strategy cells use to limit the toxicity of excess cytoplasmic amino acids.

P539/B552

Bap31 Regulates Mitochondrial Function in Er-mitochondria Contact Sites.

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The endoplasmic reticulum (ER) and mitochondria are essential organelles responsible for various cellular functions and are key components of cellular stress responsiveness. They are also hosts to an array of biological reactions that are critical for the survival and homeostatic adaptation of cells. Advances in cellular visualization technologies have revealed details regarding the organelle structure; specifically, the ER comprises large membrane-bound compartments, with a network extending throughout the cytoplasm and a membrane subdomain, the mitochondria-associated ER membrane, which is a highly conserved feature of eukaryotic cells, and appears to be in close contact with the mitochondria. Increasing evidence suggests that ER-mitochondria contact sites serve as an important cellular signaling platform associated with several important functions, including Ca^{2+} storage, lipid biogenesis, mitochondrial division, and induction of autophagy. Thus, these contact sites are involved in the regulation of cellular biological reactions; however, their role in cellular homeostasis and stress responses remains unclear. Here, we demonstrate that the ER membrane protein, BAP31, acts as a key factor in mitochondrial homeostasis to stimulate the constitution of the mitochondrial complex I by forming an ER-mitochondrial bridging protein complex. Within this complex, BAP31 interacts with mitochondria-localized proteins, including Tom40, to stimulate the translocation of NDUFS4, the component of complex I from the cytosol to the mitochondria. Disruption of the BAP31-Tom40 complex inhibits mitochondrial complex I activity and oxygen consumption by the decreased NDUFS4 localization to the mitochondria. Thus, the BAP31-Tom40 ER-mitochondria bridging complex mediates the regulation of mitochondrial function and plays a role as a novel stress sensor, representing a mechanism for the establishment of ER-mitochondrial communication via contact sites between these organelles.

P540/B553

Mitochondrial AAA+ Proteins and Their Role in Mitochondria-ER Contact Sites.

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Mitochondria play critical roles not only in primary metabolism as a central organelle for ATP generation, but also in responding to abiotic stresses. We identified a mutation in the *MTERF18 (Mitochondrial Transcription Termination factor)/SHOT1* (*Suppressor of hot1-4 1*) gene in *Arabidopsis thaliana* that enables plants to better tolerate heat and oxidative stresses, presumably due to reduced oxidative damage, but the exact molecular mechanism of the heat tolerance is unknown. In order to reveal the stress tolerance mechanisms of *mterf18*/*shot1* mutations, it is critical to understand the molecular defects of the mutant and to identify the molecular targets of the MTERF18/SHOT1 protein. MTERF18/SHOT1, a mitochondrial matrix protein, was found to bind to membrane-spanning mitochondrial AAA+ proteins homologous to ATAD3a of humans and other multicellular eukaryotes. Arabidopsis has four ATAD3a homologues in two clades, and plants require one gene from each clade for viability. Previous studies of the topology and ATPase activity of ATAD3a suggest a role in endoplasmic reticulum-mitochondria contact sites. These sites are poorly defined in plants, and their relationship to heat stress tolerance is intriguing. To better understand ATAD3 function we have expressed and purified the soluble, matrix-located, catalytic C-terminal ATPase domain of these proteins in order to assay their ATPase activity and oligomerization states. Transgenic plants with fluorescently labelled ER and mitochondria have been generated to observe effects of the MTERF18/SHOT1 mutation

on ER-mitochondria dynamics. In addition, *in-silico* structural analyses of the four ATAD proteins are being conducted to connect their structures to putative functions based on homology to known and characterized proteins. These studies of the four ATAD3 proteins will provide insights into ER-mitochondrial contact sites in plants, and into their link to MTERF18/SHOT1 and heat stress tolerance.

P541/B554

The Interaction between EBP50 and VDAC1 Mediates Cytoprotective Signaling on Mitochondria.

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Mitochondria, the organelles controlling ATP production and cellular metabolism, play a vital role in a number of cellular stress responses by communicating with other cellular compartments, for example, mitochondria-associated membranes (MAMs), the tightly membrane contact site between mitochondria and endoplasmic reticulum (ER). We recently found a scaffold protein, Ezrin/radixin/moesin (ERM)-binding phosphoprotein 50 protein (EBP50), plays a cytoprotective role on mitochondria. Knockdown of EBP50 elicited severe mitochondrial morphological change from fusion to fission and augmented apoptotic rate upon H_2O_2-induced stress and could be rescued by overexpressing siRNA resistant construct of EBP50 in Madin-Darby Canine Kidney (MDCK) cells. Furthermore, PDZ1 domain of EBP50 interacted with one of pivotal MAMs proteins, voltage-dependent anion channel 1 (VDAC1), which is localized on outer mitochondrial membrane (OMM). The N-terminus of VDAC1 regulates its oligomerization and channel activity. Amine-terminal deletion (aa 1-27) of VDAC1 diminished EBP50-VDAC1 interaction while carboxyl-terminal deletion retained an intact association. Moreover, the central component of the translocase of outer membrane (TOM) Tom20 also participated in EBP50-VDAC1 interaction. Our study paves the way for unraveling the detailed mechanisms and provides molecular insights into how to protect mitochondria from damaging during stress response.

P542/B555

Er-mitochondria Contact Sites during Mitosis.

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Recent work demonstrates that organelles contact one another and that these close membrane contact sites (MCS) are functionally critical to maintain organelle and cellular homeostasis. MCS between mitochondria and ER (referred to as MEM as well) are some of the best characterized form hotspots for different cellular processes. Mitochondria are powerhouses for cellular energy production and play a crucial role in cell metabolism under both physiological and pathological conditions. The ER is the major site of lipid synthesis and intracellular Ca^{2+} store. The ER-Mito MCSs provide a way for lipid trafficking according to the metabolic needs of the cell, and coordinate Ca^{2+} signaling and stress responses. The ER-Mito MCSs enable efficient uptake of the high local Ca2+ concentration released from the ER into mitochondria through mitochondrial Ca2+ transporters to regulate mitochondrial function. During the cell cycle, ER and mitochondria undergo dramatic reorganization and morphological changes. The ER transforms from a mixed sheets and tubules network to almost exclusively sheets structure during mitosis and the majority of mitochondria fragment. However, the dynamics of ER-Mito MCSs during cell division are still not clear. In the current study, we use EM and a split GFP approach to study ER-Mito MCSs during mitosis. We analyze ER-Mito MCSs distribution and dynamics during mitosis. Our data

demonstrate that ER-Mito MCSs are dynamic structures that undergo active remodeling during cell division.

Lipid and Membrane Microdomains 1

P543/B556

Dietary Fatty Acids Promote Lipid Droplet Diversity through Seipin Enrichment in an Subdomain of the Endoplasmic Reticulum.

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Lipid droplets (LDs) are conserved organelles that are crucial for proper compartmentalization of fat at the subcellular level. They serve as the primary sites for storing excess neutral fat. Recent studies have yielded mechanistic insights on the biogenesis and expansion of LDs that highlighted the crucial roles of endoplasmic reticulum (ER)-LD contacts. Nonetheless, outstanding questions remain on how these contacts are specifically established and preserved. Here, we report that a subpopulation of lipid droplet is defined by metabolite-modulated targeting of the *C. elegans* seipin ortholog, SEIP-1. Mutations in seipin cause the most severe form of lipodystrophy in human. Similarly, in *C. elegans*, deficiency of SEIP-1 reduces the size of a subset of LDs while over-expression of SEIP-1 has the opposite effect. Ultrastructural analysis reveals SEIP-1 enrichment in an endoplasmic reticulum (ER) subdomain, which co-purifies with LDs. An alyses of *C. elegans and* bacterial genetic mutants indicate a requirement of polyunsaturated fatty acids (PUFAs) and microbial cyclopropane fatty acids (CFAs) for SEIP-1 enrichment, as confirmed by dietary supplementations. In mammalian cells, heterologously expressed SEIP-1 engages nascent lipid droplets and promotes their subsequent expansion in a conserved manner. Our results provide the first characterization of the *C. elegans* seipin ortholog. We propose that microbial and polyunsaturated fatty acids serve unexpected roles in regulating cellular fat storage by fostering ER-LD contacts and promoting LD diversity. In an attempt to identify additional components that distinguish sub-populations of LDs, we have conducted proteomic analysis on purified LDs that are associated with or are devoid of SEIP-1(+) peri-LD cages. Results on the initial characterization of candidate molecular markers for LD sub-populations will be reported.

P544/B557

Characterization of Cerebellar Ataxia Disease-associated Snx14 in Fatty Acid Metabolism.

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Fatty acids (FAs) are important cellular metabolites which are utilized by the cells to perform important functions, such as, generation of ATP, membrane biosynthesis, and cell signaling. An imbalance in FA processing causes them to accumulate resulting in lipotoxicity - a primary cause behind metabolic and neurological disorders. To protect cells from lipotoxic stress, excess lipids are stored in the form of lipid

droplets (LDs) which emerge from the endoplasmic reticulum (ER). How the budding of LDs from the ER is regulated is not completely understood. We showed that sorting nexin protein Snx14, implicated in cerebellar ataxia disease SCAR20, is a marker for ER-LD contacts where it promotes oleate-induced LD growth. We used proximity based APEX2 technology and structure-function analysis to show that Snx14 anchors to the ER via its N-terminal transmembrane domain and interacts with LD surface via its C-terminal amphipathic helix. Loss of Snx14 perturbs LD morphology whereas Snx14 overexpression extends ER-LD contacts and promotes LD biogenesis. To further understand the implications of Snx14 in regulating ER-LD contacts, we conducted a proteomic screen to determine the interactors of Snx14 using proximity based APEX2 method. Proximity labeling of Snx14 revealed known LD proteins as well as FA desaturase SCD1 (stearoyl-CoA desaturase-1). We validated the interaction of SCD1 with Snx14 by co-immunoprecipitation experiments. Furthermore, $SNX14^{KO}$ cells are hypersensitive to palmitic acid-induced lipotoxicity and display altered ER morphology. Also, loss of Snx14 affects SCD1 expression. Current and future studies aim to mechanistically dissect the interplay of Snx14 and SCD1 in maintaining lipid homeostasis.

P545/B558

Mechanism of Lipid Droplet/Mitochondria Contacts and Role of Perilipin 5 in Lipid Metabolism.

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The lipid droplet (LD) serves as a cellular lipid store; however recent work has highlighted the ability of LDs to regulate intracellular lipid trafficking. Perilipins are LD proteins which function as lipolytic barriers and protect the cell from lipotoxicity as well as excess reactive oxygen species. A recently characterized member of the family, Perilipin 5 (Plin5), is enriched in β-oxidizing tissues where it serves as a lipolytic barrier under basal conditions. Upon protein kinase a (PKA)-stimulated phosphorylation, however, Plin5 promotes lipolysis through recruitment of the lipase ATGL and its activator CGI-58. In addition to playing a role in lipolytic control, Plin5 contains a unique C-terminal domain which has been shown to promote contacts between LDs and mitochondria (Mito). However, the mechanism by which this occurs and the physiological significance of these contacts remains to be elucidated. Utilizing predictive modeling we have identified and generated constructs containing a minimal contact domain capable of inducing LD/Mito contacts. Overexpression of Plin5 lacking this contact domain (Plin5 CΔ) leads to accumulation of lipid droplets to the same degree as full-length Plin5, indicating that LD/Mito contacts are not required for the lipolytic barrier function of Plin5. Affinity purification-mass spectrometry utilizing Plin5 and Plin5 CΔ constructs indicates a role for previously unidentified protein interactors to mediate LD/Mito tethering. To assess the physiological roles of Plin5's functional domains in fatty acid (FA) trafficking we have utilized a fluorescent FA pulse-chase assay. Our data demonstrates that efficient FA trafficking in response to nutrient deprivation is dependent on LD/Mito contacts as well as phosphorylation of Plin5 at serine 155, a known PKA phosphorylation site. Considering the expression of Plin5 primarily in β-oxidizing tissue, we hypothesize that LD/Mito contacts are not primarily involved in FA storage but rather enhance the transfer of FAs from LD to mitochondria under physiological conditions that stimulate PKA activity, leading to increased FA oxidation.

P546/B559

Kinesin Acts as a Tether between Lipid Droplets and the Endoplasmic Reticulum.

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Lipid Droplets (LDs) are dynamic cellular organelles specialized in storing and supplying lipids for metabolic processes. Mutations in proteins associated with LDs result in pathologies such as non-alcoholic fatty liver disease and congenital lipodystrophies. A recent report from our lab suggested that the kinesin-1 motor transports LDs to the smooth endoplasmic reticulum (sER) in hepatocytes, where-after lipids inside LDs can be channeled for production of lipoproteins at the sER[1]. We subsequently also showed that this channeling is controlled in the liver through Insulin signaling in a manner that maintains lipid homeostasis in blood across fed/fasted states[2]. Here we have explored the interaction between LDs, kinesin, and ER in non-hepatocyte (COS7) cells. The tubular ER network can be visualized easily along with LDs in live COS7 cells, making them a good system to study LD-ER interactions and dynamics. LDs are seen to move along the ER in a restricted manner in normal cells. However, contrary to expectations, loss of kinesin-1 leads to a dramatic increase in motion of the LDs along the ER tubules. Interestingly, the same effect is not observed upon dynein inhibition. Together, these results lead us to hypothesize that kinesin acts as a "tether" to keep LDs close to the ER. Optical trapping experiments show enhanced force-generation events that upon loss of kinesin, and these events are now ascribed to the dynein motor. Rescue experiments with truncated forms of kinesin-1 show that kinesin does not use its ATPase activity in performing this tethering function. Kinesin-1 knockdown leads to aberrant trafficking of proteins and lipids between LDs and the ER. Interestingly, loss of kinesin-1 phenocopies the knockout of Seipin, a gene involved in a severe form of congenital lipodystrophy. We are currently investigating whether kinesin facilitates the maintenance of ER-LD contacts via interaction with Seipin. Our work opens the possibility that kinesin-1 on LDs may function as a motor or a tether depending on yet-to-be-understood cellular requirements. **References:-** 1- Kinesin dependent Mechanism for Controlling Triglyceride Secretion from the Liver, Priyanka Rai et al, PNAS 114, 12958-12963 (2017) 2- Insulin activates Intracellular transport of Lipid Droplets to release Triglycerides from the Liver, Mukesh Kumar et al, Journal of Cell Biology (2019, in press)

P547/B560

Apolipoprotein E Is an Astrocyte Lipid Droplet Protein at the Intersection of Lipid Storage and Secretion.

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Neurons require large amounts of lipid to facilitate the development of mature synapses, but do not produce enough to meet these requirements. Lipids synthesized in other organs and packaged into circulating lipoproteins cannot pass through the blood brain barrier. Therefore, a majority of brain lipids are made in situ in the brain. Astrocytes, a type of glial cell involved in regulating synapse formation, maturation, and elimination, synthesize lipids and supply them to neurons via secreted lipoprotein particles or store them in cytoplasmic lipid droplets. The primary protein component of lipoprotein particles secreted by astrocytes is Apolipoprotein E (ApoE), the major genetic risk factor for late-onset Alzheimer's disease. Previously, a proximity labelling-based proteomics screen identified ApoE as a candidate protein found on lipid droplets, intracellular organelles that store esterified lipids. We hypothesize that ApoE can localize to lipid droplets in astrocytes and is involved in the regulation of lipid storage. To test whether ApoE localizes to lipid droplets (LDs), we expressed a fluorescently-tagged

ApoE construct in primary rat astrocytes. We found that ApoE localizes to LDs in some cells, while in others it is enriched in the Golgi and highly mobile vesicles. This localization was confirmed by immunostaining of endogenous ApoE. The C-terminal domain of ApoE mediates lipid droplet association, while the N-terminal domain is required for ApoE to subvert translocation into the ER. ApoE localization to LDs is dependent on nutrient availability, and correlates with increased neutral lipid storage. Together, these results suggest that ApoE plays a novel role in lipid storage and metabolism in a subset of astrocytes. We propose that ApoE is at the intersection of lipid storage and secretion, and can shift the balance between the two in response to changing environmental or developmental conditions.

P548/B561

The Roles of Pex30 and Family Members in Organelle Biogenesis at the Endoplasmic Reticulum.

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Lipid droplets (LDs) and peroxisomes are ubiquitous organelles with central roles in eukaryotic cellular metabolism. LD biogenesis and formation of pre-peroxisomal vesicles (PPVs), which are involved in peroxisomes biogenesis, occur at the same endoplasmic reticulum (ER) sites and involves cooperation between the seipin complex and Pex30p. Intriguingly, Pex30p shares the same domain architecture with four other proteins - Pex28p, Pex29p, Pex31p and Pex32p - constituting the family of the dysferlin domain-containing peroxins. However, whether these proteins are redundant or have distinct functions is unclear. To study the function of these proteins, we first endogenously tagged them and analysed their subcellular location under different conditions and genetic backgrounds. All of the Pex30-like proteins localise to the ER and form-puncta-like structure, and a fraction of these puncta correspond to ER-to-peroxisome contact sites (EPCONS). Additionally, our data suggest that the Pex30p-like proteins localise to multiple but distinct organelle contact sites in response to different physiological contexts. In turns, Pex30p-like proteins recruit Pex30p to these contact sites. In addition, we also observe that Pex30p is required for stability of Pex30-like proteins at these contact sites. These data suggest that Pex30p and Pex30p-like proteins form pairwise functional complexes at the interface of the ER with other organelles. We propose that Pex30p family of proteins are a general regulator of organelle contact sites with important roles in membrane and lipid homeostasis.

P549/B562

Lipid Storage in Starved *Tetrahymena Thermophila*.

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Lipid droplets are increasingly recognized as important and dynamic organelles in all cells. The pathways contributing to the formation and degradation of lipid droplets have been well-studied in a variety of organisms, including mouse, human, roundworm, fruit flies, and yeast. Our experiments aim to determine the cellular pathways that contribute to lipid droplet homeostasis in *Tetrahymena thermophila,* a single-celled ciliate. In this study, we demonstrate increased neutral lipid storage in *Tetrahymena* following starvation. Lipid droplet number and size increase within 3 hours of starvation, remain elevated for an additional 72 hours and then begin to decline. Pulse-chase experiments using a fluorescent fatty acid analog (Red C12) show that lipid trafficking to lipid droplets in starved *Tetrahymena* occurs along a similar time frame. We hypothesize that *Tetrahymena* mobilizes lipids in lipid droplets to serve as substrates for beta-oxidation. However, our results suggest that lipophagy does

not play a role in this process. Monodansylcadaverine, a fluorescent label for autophagosomes, fails to colocalize with lipid droplets in starved *Tetrahymena.* Future studies will use inhibitors of cytosolic lipases and/or lipophagy to investigate the pathways of lipid droplet metabolism in *Tetrahymena.* We have also identified several novel lipid droplet-associated proteins from starved *Tetrahymena* following lipid droplet isolation, SDS-PAGE and mass spectrometry. Ongoing analysis of these proteins will provide further insight into mechanisms of lipid storage in starved *Tetrahymena thermophila.*

P550/B563

The Lipid Droplet Regulator Seipin Mediates Fatty Acid Homeostasis Necessary for the Formation of the Permeability Barrier in the *Caenorhabditis Elegans* Embryo.

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The organelle lipid droplet (LD) maintains lipid homeostasis through storing and metabolizing fats in response to diverse stimuli. How these activities connect to animal growth and development are poorly understood. Seipin, crucial for adipocyte development in human, is an evolutionarily conserved protein regulating LD assembly. We found that *Caenorhabditis elegans* deleted for seipin ortholog, SEIP-1, reduced hatch ratio. The defect is largely due to the failure in synthesizing the permeability barrier that protects developing embryos from penetration of small molecules. SEIP-1 is widely expressed, enriched in major fat storage tissues as well as oocyte and embryos. SEIP-1 deletion animals accumulate aberrant LDs, consistent with its crucial role in LD size maintenance. Lipidomic analyses reveal that the SEIP-1 deleted embryo has altered fatty acid (FA) homeostasis with a significant reduction in polyunsaturated FA (PUFA) levels, consistent with the PUFA biosynthetic gene expression pattern. Interestingly, dietary supplementation with selected PUFA, namely GLA and DGLA, largely relieves the reproductive defect of the SEIP-1 deletion worm via the restoration of permeability barrier during early embryogenesis. Together, we propose that SEIP-1 maintains fatty acid homeostasis in order to orchestrate proper PUFA supplies for permeability barrier synthesis during early *C. elegans* development.

P551/B564

Spastin Tethers Lipid Droplets to Peroxisomes and Directs Fatty Acid Trafficking through ESCRT-III.

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Lipid droplets (LDs) are neutral lipid storage organelles that transfer lipids to various organelles including peroxisomes. Here, we show that the hereditary spastic paraplegia protein M1 Spastin, a membrane-bound AAA ATPase found on LDs, coordinates fatty acid (FA) trafficking from LDs to peroxisomes through two interrelated mechanisms. First, M1 Spastin forms a tethering complex with peroxisomal ABCD1 to promote LD-peroxisome contact formation. Second, M1 Spastin recruits the membrane-shaping ESCRT-III proteins IST1 and CHMP1B to LDs via its MIT domain to facilitate LD-to-peroxisome FA trafficking, possibly through IST1- and CHMP1B-dependent modifications in LD membrane morphology. Furthermore, LD-to-peroxisome FA trafficking mediated by M1 Spastin is required to relieve LDs of lipid peroxidation. M1 Spastin's dual roles in tethering LDs to peroxisomes and in recruiting ESCRT-III

components to LD-peroxisome contact sites for FA trafficking may underlie the pathogenesis of diseases associated with defective FA metabolism in LDs and peroxisomes.

P552/B565

Regulation and Compartmentalization of Fatty Acid Metabolism at Membrane Contact Sites.

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Fatty acids (FA) are toxic to cells and thus sequestered in the form of triacylglycerides in cytoplasmic organelles called lipid droplets (LD) that bud from the endoplasmic reticulum (ER). We recently showed that, in response to nutrient stress, yeast LDs cluster adjacent to the vacuole/lysosome, but how this LD accumulation is spatially coordinated remains unknown. We report that ER-vacuole tether Mdm1 spatially regulates LD biogenesis at ER-vacuole contacts to maintain ER lipid homeostasis and protect the cell from lipotoxicity. Mdm1 binds to LDs and free FAs via its uncharacterized PX-Associated (PXA) domain, and co-enriches with fatty acyl-CoA ligase Faa1 at LD bud sites. Consistent with this, loss of *MDM1* perturbs free FA activation and subsequent TAG generation, elevating cellular FAs that perturbs ER morphology and sensitizes yeast to FA-induced lipotoxicity. Our data support a model where Mdm1 organizes free FA activation adjacent to the vacuole to promote LD production in response to stress. This maintains ER lipid homeostasis by efficiently segregating away toxic FAs into LDs. These findings establish a functional significance for organelle contacts in organizing FA metabolism and lipid flux. We are currently investigating the mechanism by which Mdm1 coordinates the metabolic fates of free FAs at organelle contact sites through a combination of in *vitro* reconstitution, global lipidomics, and state-of-the-art cryogenic focused ion beam (Cryo-FIB) milling and electron microscopy.

P553/B566

Phase-partitioning of Vacuole Membrane Directs Inter-organellar Sterol Trafficking to Coordinate Mitochondrial Development and Energy Metabolism with Macro-autophagy.

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Cellular ability to switch from aerobic fermentation to mitochondrial oxidative phosphorylation is the key underlying survival mechanism for many organisms during glucose starvation. However, how cells drive this metabolic transition remains unclear. Using a yeast model system, we demonstrate that to sustain mitochondrial respiration, by which lipid is now utilized as the major energy source, starved cells activate a distinct inter-organellar lipid pipeline to mitochondria from vacuole/lysosome via endoplasmic reticulum (ER). We find that macro-autophagy initiates this new lipid route by rapidly relocating endosomal membrane compartments to the vacuole. This prompted vacuole membrane phase separation, generating ergosterol-enriched liquid-ordered membrane-like (Lo) domains. Through the Lo domain, vacuole-ER contacts formed, and vacuolar ergosterols cycled back to ER for mitochondria and lipid droplet (LD) developments. With a control of vacuolar ergosterol pools by LD sequestration of excessive sterol lipids, the Lo domains further differentiated, and vacuolar LD recruitment and digestion began to work in play for recycling of LD-stored fat molecules. Our findings that the Lo differentiation occurs only when mitochondrial respiration promotes macro-autophagy suggested a feedback interplay between mitochondria and vacuole during the remobilization of the needed substitute energy source from LDs. Taken together, our results reveal an unknown organellar mechanism that mediates metabolic

reprogramming in glucose-starved cells, and highlight the importance of inter-organellar communications for cell and organism's survival under metabolic stress.

P554/B567

Extended Synaptotagmin Regulates Plasma Membrane-endoplasmic Reticulum Contact Site Structure and Lipid Transfer Function in *Vivo*.

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Inter-organelle communication by membranes positioned in close proximity is proposed to occur at Membrane Contact Sites (MCS). MCS between the plasma membrane (PM) and endoplasmic reticulum (ER) are thought to support localized biochemical events including lipid transfer between these membranes and several proteins that localize to ER-PM contact sites have been identified. The extended synaptotagmins (Esyt) are proteins localized to MCS in both yeast and cultured mammalian cells. Several models of their localization and function at ER-PM contact sites are described. However, loss of all three Esyts in yeast (*tcb1, 2 and 3*) or mouse (*Esyt1, 2 and 3*) shows minimal phenotypes raising questions about the requirement of Esyt for cell physiology in *vivo. In Drosophila* photoreceptors, G-protein coupled phosphatidylinositol 4,5 bisphosphate (PIP_2) turnover is required for phototransduction. The phosphatidylinositol transfer protein (RDGB) is localized to ER-PM contact sites in photoreceptors and is required to support normal light responses in *vivo*. Depletion of RDGB or its mis-localization away from MCS results in a reduced light response and retinal degeneration. Using CRISPR/Cas9 genome engineering, we created a protein null mutant of the only extended synaptotagmin in *Drosophila (dEsyt)* and studied its impact on photoreceptor structure and function. Using electron microscopy analysis, we find that *dEsyt* is required to maintain the structure and function of ER-PM MCS in *vivo*. Loss of dEsyt results in progressive mislocalization of RDGB protein during illumination ultimately leading to retinal degeneration. Importantly, loss of *dEsyt* enhanced the in *vivo* phenotypes of $rdgB^{9}$, a hypomorphic allele with reduced levels of RDGB protein. $rdgB^{9};dEsyt^{KO}$ double mutants show an enhanced rate of retinal degeneration, reduced light responses and slower rates of plasma membrane PIP_2 resynthesis compared to $rdgB^{9}$. Thus, we demonstrate that dEsyt function is required to support ER-PM contact site structure of photoreceptors and the function of RDGB at this location in *vivo*.

P555/B568

Ca2+-dependent Regulation and Lipid Transport Mechanism of Extended Synaptotagmin 1.

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The extended synaptotagmins (E-Syts) are intrinsic membrane proteins of the endoplasmic reticulum (ER) that bind the plasma membrane (PM) via C2 domains and transport lipids between the two membranes via SMP domains. Of the five C2 domains of E-Syt1, only C2A and C2C contain Ca2+-binding sites. Using liposome-based assays, we showed that Ca2+ binding to C2C promotes E-Syt1-mediated membrane tethering by releasing an autoinhibitory interaction that prevents C2E from binding to PI(4,5)P2-rich membranes. Additionally, Ca2+ binding to C2A enables lipid transport by releasing a distinct and charge-based autoinhibitory interaction between this domain and the SMP domain. To gain insight into whether the SMP domains can transport lipids between two adjacent bilayer by shuttling

between them, rather than by directly bridging the two bilayers to allow sliding of lipids within its hydrophobic groove, we developed DNA-origami nanostructures that organize size-defined liposomes at precise distances and allow lipid transfer between them. Pairs of DNA-ring-templated donor and acceptor liposomes were docked through DNA pillars, which determined their distance. The SMP domain was anchored to donor liposomes via an unstructured linker, and lipid transfer was assessed via a FRET-based assay. We found that lipid transfer can occur over distances that exceed the length of an SMP dimer. Thus, we propose a model in which Ca2+ binding reverses the autoinhibited state of E-Syt1 to promote membrane tethering and lipid transport via the shuttling of its SMP domain between the two adjacent membranes.

P556/B569

PDZD8 Mediates a Rab7 Dependent Interaction of the Endoplasmic Reticulum with Late Endosomes and Lysosomes.

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The endoplasmic reticulum (ER) is the subcellular compartment where most membrane lipids are synthesized and where lipid metabolites generated in other membranes are returned to be reutilized. Lipid flux from and to the ER is partially mediated by proteins that are localized at sites of close appositions between its membrane and those of other membranous organelles and that contain lipid transport modules. The SMP domain is one such module. Proteins harboring this domain also function as tethers between the two adjacent membranes. Here we focus on PDZD8, an SMP domain containing protein, previously reported to act at contacts between the ER and mitochondria (PMID: 29097544). PDZD8 is an ER-resident protein inserted in the ER membrane through an N-terminal transmembrane domain. The cytosolic portion of PDZD8 contains the SMP domain followed by a PDZ domain, a C1 domain and a C-terminal coiled-coil region. Using confocal microscopy, we have now found that both PDZD8-EGFP transfected in fibroblastic cells and PDZD8 tagged at the endogenous locus in Neuro2A cells, are not only present throughout the ER but also enriched at regions of the ER that contact a subset of late endosomes/lysosomes. Recruitment of PDZD8 to these vesicles is mediated by its interaction with the small GTPase Rab7. The interaction is highly dependent on the GTP-bound state of Rab7 and is mediated by the most C-terminal region of PDZD8 which contains a coiled-coil domain. Confocal microscopy further showed that overexpression of both PDZD8 and Rab7 (more so of the mutant constitutively active Rab7) induces massive enwrapping by the ER of late endosomes/lysosomes with PDZD8 being concentrated at the interface between the ER and these organelles. Electron microscopy confirmed the occurrence of massive and very tight appositions between the ER and late endosomes/lysosomes in these cells. As Rab7 is a key modulator of endosomal maturation, these findings raise the possibility that the lipid transfer activity of PDZD8 may have a role in this transition. It would be of interest to determine whether the previously reported defect in mitochondrial function resulting from the absence of PDZD8 may be an indirect effect of late endosome/lysosome dysfunction (PMID: 29097544).

P557/B570

Metabolic Integration of Signaling and Housekeeping Pools of Phosphoinositides between the ER and Plasma Membrane.

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Phospholipase C (PLC)-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P_2) is a major signaling modality in all metazoan organisms. During prolonged stimulation, the limited plasma membrane (PM) pool of PI(4,5)P_2 is replenished using phosphatidylinositol (PI) that is synthesized in the endoplasmic reticulum (ER). PI synthesis is strongly stimulated during PLC activation and it is believed that this increase is the result of recycling both hydrolytic products of PI(4,5)P_2 hydrolysis, inositol 1,4,5-trisphopshate and diacylglycerol (DG). An important step in the recycling process is the conversion of DG to phosphatidic acid (PA) within the PM and the subsequent transport of PA from the PM to the ER. PI is synthesized from PA by two enzymatic reactions that occur exclusively in the ER, with cytidine diphosphate-DG (CDP-DG) being a short-lived intermediate. In the ER, there are several sources of PA, including PA derived from *de novo* synthesis, phospholipase D (PLD)-mediated phosphatidylcholine (PC) hydrolysis or from the phosphorylation of ER-localized DG by DG-kinases. To what extent these different sources of PA can be used for PI synthesis during PLC activation is poorly understood. Here, we used a combination of isotope labeling and lipidomics analyses in agonist-stimulated HEK293 cells to investigate the metabolic isolation of the various sources of PA that are used to increase PI synthesis. Our studies show that PLC activation primarily initiates the recycling of the PM-derived hydrolytic products from PI(4,5)P_2 breakdown, but also enhances *de novo* PA synthesis and its conversion into PI. Notably, the unsaturated PI(4,5)P_2 pools are resynthesized significantly faster than the more saturated ones suggesting a slower integration of saturated PI(4,5)P_2 pools into the signaling pool during prolonged PLC activation. These findings have significant implications for understanding the recycling and distribution of PI lipids between the ER and other organelles during PLC activation while also shedding light on the fatty acid side chain remodeling that occurs during these processes.

P558/B571

***Grasp55*$^{-/-}$ Mice Display Impaired Fat Absorption and Resistance to High-fat Diet-induced Obesity.**

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The Golgi apparatus plays a central role in the intracellular transport of macromolecules. However, molecular mechanisms of Golgi-mediated lipid transport remain poorly understood. Here, we show that genetic inactivation of the Golgi-resident protein GRASP55 in mice reduces whole-body fat mass via impaired intestinal fat absorption and evokes resistance to the weight gain induced by a high-fat diet. Mechanistic analyses revealed that GRASP55 participates in the Golgi-mediated lipid droplet (LD) targeting of some LD-associated lipases, such as ATGL and MGL, which is required for sustained lipid supply for chylomicron assembly and secretion. Consequently, GRASP55 deficiency led to reduced chylomicron secretion and abnormally large LD formation in intestinal epithelial cells upon exogenous lipid challenge. Notably, silencing of dGrasp in Drosophila caused similar defects of lipid accumulation in the midgut. These results highlight the importance of the Golgi complex in cellular lipid regulation, which is evolutionary conserved, and uncover potential therapeutic targets for obesity-associated diseases.

P559/B572

Protein Engineering of an Improved Pair of Magnets Photodimerizer Proteins, and Their Use to Rapidly and Locally Reconstitute VAP-dependent Interactions between the Endoplasmic Reticulum, Golgi, and Endosomes.

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A distinctive feature of eukaryotic cells is the partitioning of the cytoplasm into membrane-enclosed organelles to orchestrate and separate cellular processes. Organelles typically communicate with one another through vesicular transport or via direct membrane contacts. The VAMP-associated proteins (VAPs), comprising VAPA and VAPB, are tail-anchored ER membrane proteins that tether the ER to other membranes via interactions with membrane proteins in other organelles. VAPs contain: 1) a cytosolic N-terminal MSP domain, which binds proteins containing the so-called FFAT motif, 2) a coiled-coil region responsible for homo/heterodimerization, and 3) a C-terminal membrane anchor. VAP-driven ER-organelle contacts play a multiplicity of functions, including trafficking of phosphatidylinositol 4-phosphate (PI4P) from other organelles to the ER for degradation by the phosphatidylinositol phosphatase enzyme Sac1. This transport activity is mediated by oxysterol-binding protein-related proteins (ORPs) that bind VAP via an FFAT motif and other membranes via a PH domain. Accordingly, HeLa cells lacking VAP exhibit several defects, including abnormally high accumulation of PI4P in the Golgi complex and endosomes. Detailed exploration of the role of VAP in cell physiology would benefit from the possibility of reversibly turning on and off its tethering function. To achieve this goal, we first engineered an improved blue light-dependent dimerization system starting from the charged-complementary dimerization pair of the LOV-domain Magnets proteins. Importantly, Magnets do not require exogenous cofactors, hetero- rather than homo-oligomerize, are very small (147 aa), can be fused to either terminus of a protein, are functional when tethered to intracellular membranes, and require simultaneous photoactivation to achieve dimerization, which enables high spatial confinement with a single wavelength of light. A serious drawback of Magnets for studies of mammalian cells, however, is their low temperature (28^{o}C) requirement for proper folding. To overcome this limitation, we engineered an improved Magnets pair that fold at mammalian physiological temperatures. We used this tool to rapidly and reversibly recruit of VAPB to the ER membrane in VAPA/VAPB double-knockout cells to acutely reconstitute VAP function. Exposure of these cells to blue light reduced PI4P accumulation on endosomes and Golgi within tens of seconds, validating the use of the improved Magnets to rapidly manipulate subcellular functions in mammalian cells with high temporal and spatial precision. This improved Magnets pair will be useful for creating many optogenetic reagents.

P560/B573

Loss of Lysosomal Npc1 Function Increases PtdIns4P to Sustain ER to Golgi Cholesterol Transfer.

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Cells utilize vesicular and non-vesicular transport mechanisms to transport lipids to target organelles. Non-vesicular lipid transport is mediated by transfer proteins that tether the gap between two closely apposed organelle membrane contact sites. At contact sites between Trans-Golgi network (TGN) and Endoplasmic reticulum (ER), oxysterol-binding protein (OSBP) mediates cholesterol/ PtdIns4P exchange. In health, TGN PtdIns4P is transported down its concentration gradient to ER, fueling the

countertransport cycle of cholesterol to TGN against its steep concentration gradient. Maintaining the PtdIns4P gradient and thus perpetuating this exchange cycle, are PtdIns4P kinases on the TGN and a PtdIns4P phosphatase (Sac1) on the ER. Consequently, this cholesterol/PtdIns4P exchange contributes to the establishment of a cholesterol gradient between organelles of the secretory pathway, essential for protein trafficking to the plasma membrane (PM). Despite this knowledge, little is known about how this molecular choreography is disrupted during pathophysiological conditions. In Niemann-Pick Type C1 (NPC1) disease, a pathological condition that leads to hyperexcitability and neurodegeneration, there is an alteration of lysosomal cholesterol homeostasis. Here we show that in models of NPC1 disease, there is a rearrangement of molecular components of the cholesterol/PtdIns4P cycle. Using a combination of mass-spectrometry, super-resolution imaging, and electrophysiology, we have discovered that inhibition or disease-causing mutations in the lysosomal transporter Niemann-Pick Type C1 result in a significant elevation in Golgi PtdIns4P. The increase in TGN PtdIns4P is not due to a slower rate of OSBP-mediated PtdIns4P transfer, or increased protein levels of PI4K isoforms. Instead, the increase in TGN PtdIns4P levels is due to spatial redistribution of PI4KIIα and PI4KIIIβ isoforms. Concurrent with the enhanced recruitment of PI4KIIIβ to the TGN is a significant accumulation of endogenous ER Sac1 at the contact site between the ER and TGN. The parallel reorganization of PI4KIIIβ and Sac1 ensures the maintenance of a steep PtdIns4P gradient that strengthens cholesterol transport to the Golgi. We also find that a downstream consequence for the increased cholesterol/ PtdIns4P exchange in NPC1 disease, is a dysregulation of vesicular trafficking of the AMPA Receptor Subunit GluA1. Physiologically, altered GluA1 subunit density at the PM aberrantly modifies the influx of calcium into neurons and may contribute to neuronal cell death in NPC1 disease. We propose that lysosomal NPC1-mediated cholesterol efflux modulates the molecular organization of TGN-ER contact sites to influence the driving force for cholesterol transport in health and disease.

P561/B574

The Interaction between Non-fusogenic Sec22b-syntaxin Complexes and Extended-synaptotagmins Promotes Neurite Growth and Ramification.

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Axons and dendrites are long and often ramified neurites that need particularly intense plasma membrane expansion during the development of the nervous system. Neurite growth depends on non-fusogenic Sec22b-Syntaxin1 SNARE complexes at endoplasmic reticulum (ER)-plasma membrane (PM) contacts. Here we show that Sec22b interacts with the endoplasmic reticulum lipid transfer proteins Extended-Synaptotagmins (E-Syts) and this interaction depends on the Longin domain of Sec22b. Overexpression of E-Syts stabilizes Sec22b-Stx association, whereas silencing of E-Syts has the opposite effect. Overexpression of wild-type E-Syt2, but not mutants unable to transfer lipids or attach to the ER, increase the formation of axonal filopodia and ramification of neurites in developing neurons. This effect is inhibited by a clostridial neurotoxin cleaving Stx1, expression of Sec22b Longin domain and a Sec22b mutant with extended linker between SNARE and transmembrane domains. We conclude that Sec22b-Stx1 ER-PM contact sites contribute to PM expansion by interacting with E-Syts.

P562/B575

Phosphatidylinositol-4,5-bisphosphateRegeneration during Muscarinic Receptor 1 Stimulation.

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Phosphatidylinositol 4,5-bisphosphate (PtdIns[4,5]P_2) participate many cellular functions. For example, regulation of ion channels, regulation of endo-, exocytosis, and precursor of Inositol-1,4,5-triphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG). With G alpha q protein-coupled receptor (GqPCR) expressed cells, phosphatidylinositol 4-phosphate (PtdIns4*P*) and PtdIns[4,5]P_2 are depleted in ~ 20 s by Oxotremorin (Oxo-M), but if we leave the agonist on continuously for 10 min, the PtdIns[4,5]P_2 is recovered. Indicator of Ins(1,4,5)P_3 and DAG, products of PtdIns[4,5]P_2 hydrolysis, increased continuously in stimulation of GqPCR. Additionally, by the mathematical computer model, we found acceleration of PtdIns[4,5]P_2 regeneration model is matched with actual data. As a results, PtdIns[4,5]P_2 regeneration is accelerated during GqPCR stimulation. PtdIns4*P* is a precursor of PtdIns[4,5]P_2. Therefore PtdIns 4-kinases, enzymes that phosphorylate Phosphatidylinositol (PtdIns) to PtdIns4*P*, are key molecules for regeneration. When we blocked PtdIns 4-kinases by pharmacological method, we found *PI4KA* (PI4KIIIα) is a key enzyme to PtdIns[4,5]P_2 recovery. Additionally, the Golgi PtdIns4*P* is major contributor for regeneration of PtdIns[4,5]P_2, not membrane PtdIns4*P*

P563/B576

Yeast Phosphatidylinositol Transfer Proteins Pdr16 and Pdr17: Similar, Yet Different.

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The yeast *Saccharomyces cerevisiae* possess 6 proteins classified as phosphatidylinositol transfer proteins (PITPs). Two of the yeast PITPs, Pdr16p (also called Sfh3p) and Pdr17p (also known as Sfh4p, PstB2p or Iss1p) are 49% identical and 75% similar. Pdr16p regulates lipid droplet utilization, clearance of cytosolic inclusion bodies, and is required for resistance to azole antifungals. Pdr17p is an essential component of a complex required for transport of phosphatidylserine (PS) between the ER and the Golgi apparatus/endosomes. To study the function of these two proteins in more detail we examined the role of phosphatidylinositol binding by these lipid transfer proteins. We also investigated the role of Pdr17p in PS transfer between the ER and the Golgi apparatus/endosomes using random mutagenesis approach. We found, using Pdr16p and Pdr17p mutants defective in PI binding, that for the proper function of Pdr16p in ensuring resistance to azole antifungals both high affinity PI binding and the ability to transfer PI between membranes must be retained. In contrast, Pdr17p high affinity PI binding is dispensable for Pdr17p function in a complex that is required to transfer PS from the ER to the Golgi apparatus/endosomes. We observed that PI binding defective Pdr17p mutant is not able to complement a defective Sec14p or a missing Pdr16p. This feature enabled the preparation of Pdr17^{Ser174Pro} mutant specifically defective in PS transfer from the ER to the non-mitochondrial site of PS decarboxylation. This mutant is defective in this process most likely due to its inability to interact with PS decarboxylase 2. Our results provide evidence that Pdr16p fulfills role(s) in which the ability to bind and transfer PI is required. In contrast, Pdr17p adapted to a different role which does not require high affinity PI binding, although the protein retains the capacity to transfer PI in *vitro.* Importantly, Pdr16p and Pdr17p bind also cholesterol in addition to PI in an assay using permeabilized HL-60 cells. Our results underscore the need

to follow multiple parameters of lipid transfer proteins when studying these proteins. This research was supported by the Scientific Grant Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic and the SAS grant 2/0111/15, the Slovak Research and Development Agency contract No. APVV-15-0654, and the British Heart Foundation grant FS/15/73/31672.

P564/B577

Insulin Controls Kinesin-dependent an terograde Transport of Lipid Droplets to Release Triglycerides from Liver.

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Despite massive fluctuations in its internal triglyceride content, liver continues to secrete triglyceride under tight homeostatic control. This fine-tuned lipid homeostasis is most evident during fasting as triglyceride level increases by many folds in the liver, but serum triglyceride levels barely fluctuate. Unfortunately, the molecular mechanism underlying this lipid homeostasis in the liver remain largely unclear. Moreover, this buffering ability of the liver is essential to prevent systemic hyperlipidemia across our daily feeding-fasting cycles. We have uncovered a novel cellular mechanism involved in regulating triglyceride secretion from the liver across feeding-fasting cycles. A molecular interplay between a key metabolic-hormone (Insulin), the smallest phospholipid (phosphatidic acid, PA) and a molecular motor (kinesin-1) maintains triglyceride homeostasis in the liver. In fed state, insulin activates ARF1-GTPase on lipid droplets (LDs, triglyceride depot) that generate reactive LDs and also recruits phospholipase D1 (PLD1) on its membrane. The recruited PLD1 hydrolyses phosphatidylcholine (PC) into PA, which in turn recruits Kinesin-1 motor protein on LDs and translocated them to cortically located smooth endoplasmic reticulum (sER). The LD-sER contacts at the cell periphery ensure the delivery of LD-triglycerides to the lumen of sER for lipo-protein (VLDL) assembly. During fasting *i.e*. In absence of insulin signal, this entire pathway is downregulated and the triglyceride secretion is tempered to maintain the serum triglyceride level constant. Pharmaceutical or molecular inhibition of this pathway at multiple levels, results in slow release of triglycerides from hepatocytes. A targeted peptide-based cell-therapy also diminishes triglyceride secretion from hepatocytes without any apparent effect on intracellular organelle distribution and cell viability. In a nutshell, our work revealed a fundamental cellular mechanism to establish systemic lipid homeostasis and multiple molecular targets against hyperlipidemia like lipid-centric disorders. **References:** Kumar M, Ojha S, Rai P, Joshi A, Kamat S S and Mallik R. 2019. Insulin activates Intracellular transport of Lipid Droplets to release Triglyceride from the Liver. *Journal of Cell Biology*. (In Press) Rai P, Kumar M, Sharma G, Barak P, Das S, Kamat S S and Mallik R. 2017. Kinesin-dependent mechanism for controlling triglyceride secretion from the liver. Proc. Natl. Acad. Sci. U. S. A. 114:12958-12963.

P565/B578

Some Lipid Droplet Biogenesis Does Not Require Seipin Or Fat Storage-inducing Transmembrane (fitm2) Proteins.

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Lipid droplets (LDs) are ubiquitous lipid storage organelles that play critical roles in lipid homeostasis. LDs are made up of a core of neutral lipids, primarily triacylglycerols (TAGs), and sterol esters (SEs), surrounded by a phospholipid monolayer. Other neutral lipids, such as retinyl esters, can also be stored in LDs. LD biogenesis occurs in the ER, where neutral lipids are synthesized, but the mechanism is not well understood. We investigated how the type of neutral lipid stored in LDs affects biogenesis. We generated a *S. cerevisiae* strain that cannot produce endogenous neutral lipids (TAGs and SEs) and instead produces retinyl esters. These cells produced LDs that contain only retinyl esters and the LDs are indistinguishable, by electron microscopy, from endogenous LDs. We next investigated whether proteins known to play roles in endogenous LD biogenesis are also necessary for generation of retinyl ester LDs. We found that retinyl ester LD biogenesis does not require seipin or fat storage-inducing transmembrane protein 2 (FITM2) proteins, unlike endogenous LDs. Our findings suggest that these proteins are necessary for the biogenesis of LDs that contain primarily TAGs but not LDs containing other neutral lipids.

P566/B579

Drosophila Adipocytes Organize Their Lipid Droplets into Spatially and Functionally Distinct Subpopulations.

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Adipocytes store nutrients as lipid droplets (LDs), but how they organize their LD stores to balance lipid uptake, storage, and mobilization remains poorly understood. Here, using Drosophila fat body (FB) adipocytes we characterize spatially distinct LD populations that are maintained by different lipid pools. We identify peripheral LDs (pLDs) that make close contact with the plasma membrane (PM) and are maintained by lipophorin-dependent lipid trafficking. pLDs are distinct from larger cytoplasmic medial LDs (mLDs) which are maintained by FASN1-dependent de novo lipogenesis. We find that sorting nexin CG1514/Snazarus (Snz) associates with pLDs and regulates LD homeostasis at ER-PM contact sites. Loss of SNZ perturbs pLD organization whereas Snz over-expression drives LD expansion, triacylglyceride production, starvation resistance, and lifespan extension through a DESAT1-dependent pathway. We propose that Drosophila adipocytes maintain spatially distinct LD populations and identify Snz as a novel regulator of LD organization and inter-organelle crosstalk.

Kinases and phosphatases 1

P567/B581

Defining How Cdr1/nim1 Inhibits Wee1.

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Entry into mitosis initiates from a switch-like activation of cyclin-dependent kinase (Cdk1). During interphase, the conserved protein kinase Wee1 phosphorylates and inhibits Cdk1 until cells have grown to a critical size threshold. To enter into mitosis, cells require mechanisms that inhibit Wee1 and allow activation of Cdk1. In fission yeast, the related protein kinases Cdr1/Nim1 and Cdr2 act as upstream inhibitors of Wee1. Several questions have remained open regarding when, where, and how Cdr1/Nim1 and Cdr2 inhibit Wee1 in cells. Past work found that Cdr1/Nim1 phosphorylates Wee1 to inhibit its kinase activity (1-3). We found that both Cdr1/Nim1 and Cdr2 induced hyperphosphorylation of Wee1, but only Cdr1/Nim1 inhibited Wee1 kinase activity. Using both purified proteins and co-expression, we found that Cdr1/Nim1 phosphorylated a cluster of residues within the C-terminal lobe of the Wee1 kinase domain. Mutations that prevent phosphorylation of these sites caused cells to divide at a larger size and to exhibit synthetic lethality with *cdc25* mutations, consistent with overactive Wee1. Further, this inhibitory phosphorylation altered the balance of Wee1-Cdk1 reciprocal regulation to alter cell size at division. Our work provides mechanistic insight into the control system that links Wee1 activity with cell size and nutrient conditions. 1. Coleman, T. R., Tang, Z., and Dunphy, W. G. (1993) Negative regulation of the wee1 protein kinase by direct action of the nim1/cdr1 mitotic inducer. *Cell* **72**, 919-929 2. Parker, L. L., Walter, S. A., Young, P. G., and Piwnica-Worms, H. (1993) Phosphorylation and inactivation of the mitotic inhibitor Wee1 by the nim1/cdr1 kinase. *Nature* **363**, 736-738 3. Wu, L., and Russell, P. (1993) Nim1 kinase promotes mitosis by inactivating Wee1 tyrosine kinase. *Nature* **363**, 738-741

P568/B582

Cell Size Is Regulated by Phosphatidylinositol 3-phosphate in *Drosophila*.

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Regulation of cell size is a key aspect of growth and development in metazoans. Growth control requires the integration of nutritional and hormonal cues and many of these are transduced by mTORC1 through its output S6K kinase, an essential modulator of protein synthesis and cell growth. It is reported that in *Drosophila*, phosphatidylinositol 5 phosphate 4-kinase (dPIP4K) regulates mTORC1 activity and cell growth. In *Drosophila* PIP4K mutants (*dPIP4K*^{29}), both mTORC1 activity and cell size are reduced. dPIP4K can phosphorylate both phosphatidylinositol 5-phosphate (PI5P) and phosphatidylinositol 3-phosphate (PI3P). It has previously been reported that PI5P levels are elevated in *dPIP4K*^{29}. We now report by LC-MS measurements that PI3P levels are also elevated in these mutants raising the possibility that elevations in PI3P may also regulate mTORC1 and cell growth. Consistent with this idea, overexpression of Vps34, a class III PI3Kinase that generates PI3P, reduces *Drosophila* salivary gland cell size and the co-expression of Myotubularin (dMtm), a PI3P 3-phosphatase that can reduce PI3P levels is able to rescue this reduced cell size. Finally, we found that overexpression of dMtm in *dPIP4K*^{29} was able to reverse both the reduced cell size as well as elevated PI3P levels. Our work defines PI3P acting via dPIP4K as a novel regulator of cell size.

P569/B583

Phosphoproteomic an alysis of the Mammalian Growth Cones Reveals the Novel Molecular Markers of the Growing/regenerating Axons, Specifically to Vertebrate.

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The growth cone is an essential structure formed in the developing axons for accurate neuronal network formation. However, in the mammalian growth cone behavior, these problems are poorly approached so far. We focused the phosphorylation which is the most important protein modification in the cellular signaling pathways, and performed the phosphoproteomic analysis of the growth cone membrane (GCM) prepared from the developing rodent brain, to comprehensively know the phosphorylation sites and their frequency for neuronal development. We identified 30,000 phosphopeptides derived from the 5,000 phosphorylation sites of 1,200 proteins. We have already found GAP-43, a vertebrate-specific neuronal protein, has a highly phosphorylated residue Ser96, and showed this phosphorylation as a growing/regenerating marker of axon (ref.1). In this study, we also featured two of the most abundant phosphorylated sites, Ser25 and Ser1201 of microtubule-associated protein 1B (MAP1B), which is a protein known to stabilize axonal microtubules and to be involved in axon formation. We produced the phospho-specific antibodies (Abs) against these sites, respectively, which preferentially labelled the growing axons in the embryonic brain. In the injured sciatic nerve, we found that the immunoreactivity of each Ab was increased at the injury site and extended to the distal side over the injured point. A considerably large amounts of these sites were phosphorylated by the proline-directed protein kinases such as JNK, which is known to be involved in axon growth within a wide range of animals from *C. elegans* or *Drosophila* to mammals. Bioinformatic analysis revealed that most of these sites were conserved within the vertebrates, however, were not conserved in simpler model organisms such *C. elegans* or *Drosophila*. In particular, most of the highly frequent phosphorylation sites (more than 20 times) in GCM were not conserved in *C. elegans* or *Drosophila*. We concluded that the mammalian signaling pathways in GCM are totally distinct from the model organisms.

P570/B584

Phosphatidylinositol Kinases and Phosphatases in the Enteric Protozoan Parasite *Entamoeba Histolytica*.

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Phosphatidylinositol (PtdIns) metabolism is indispensable in eukaryotes. Phosphoinositides (PIs) are phosphorylated derivatives of PtdIns and consist of seven species generated by reversible phosphorylation of the inositol moieties at the positions 3, 4, and 5. Each of the seven PIs has a unique subcellular and membrane domain distribution. In the enteric protozoan parasite *Entamoeba histolytica*, it has been previously shown that the PIs, phosphatidylinositol 3-phosphate (PtdIns3P), PtdIns(4,5)P_2, and PtdIns(3,4,5)P_3 are localized to phagosomes/phagocytic cups, plasma membrane, and phagocytic cups, respectively. The localization of these PIs in *E. histolytica* is similar to that in mammalian cells, suggesting that PIs have orthologous functions in *E. histolytica*. In contrast, the conservation of the

enzymes that metabolize PIs in this organism has not been well documented. Here, we report the full repertoire of the PI kinases and PI phosphatases found in *E. histolytica* via a genome-wide survey of the current genomic information. *E. histolytica* appears to have 10 PI kinases and 23 PI phosphatases. It has a panel of evolutionarily conserved enzymes that generate all the seven PI species. However, class II PI 3-kinases, type II PI 4-kinases, type III PI 5-phosphatases, and PI 4P-specific phosphatases are not present. Additionally, regulatory subunits of class I PI 3-kinases and PI 4-kinases have not been identified. Instead, homologs of class I PI 3-kinases and PTEN, a PI 3-phosphatase, exist as multiple isoforms, which likely reflects that elaborate signaling cascades mediated by PtdIns(3,4,5)P_3 are present in this organism. There are several enzymes that have the nuclear localization signal: one phosphatidylinositol phosphate (PIP) kinase, two PI 3-phosphatases, and one PI 5-phosphatase; this suggests that PI metabolism also has conserved roles related to nuclear functions in *E. histolytica*, as it does in model organisms.

P571/B585

Cyclic GMP-dependent Kinase Triggers Cytosolic Calcium Signaling in the Human Malaria Parasite *Plasmodium Falciparum*.

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Malaria is accountable for the death of more than 400,000 people across the continents in tropical and sub-tropical regions. The responsible pathogens belong to the *Plasmodium spp. Of* apicomplexan protozoa and among them; *P. falciparum* is the most virulent with the highest fatalities. The clinical symptoms of malaria are associated with the periodic rupture and exponential increase of parasites in the host circulation system where it uses the sequential discharge of the apical proteins in a complex signaling cascade to maintain the growth and transmission. The discharge trigger is shown to initiate by the secondary messengers and protein kinase activation. Here in this study, we used a genetic calcium marker parasite expressing PfGCamP3 to study the calcium efflux in the asexual trophozoite parasites that were intact in their natural red blood cell host. We found that inhibiting the *P. falciparum* phosphodiesterase by zaprinast induce transient calcium release from the parasite calcium store. We observed that the zaprinast induced calcium rise was abolished in cyclopiazonic acid (CPA) pretreatment, suggesting a common source. The calcium signal is specific to cGMP, as stimulating with a cell-permeant analog 8Br-cGMP abrogates the zaprinast-induced calcium response and vice-versa in *P. falciparum* parasites. When we chelate the cytosolic calcium with BAPTA-AM, zaprinast was unable to elevate cytosolic calcium indicated the possible flux from the inner source. Interestingly, zaprinast induced calcium rise was also blocked in the presence of a protein kinase G (PKG) inhibitor compound-1. To find out the further downstream sequence, we used a phospholipase C (PLC) inhibitor, which interrupted the calcium efflux upon zaprinast induction strongly indicating the PLC-IP_3 mediated calcium release, however, no canonical IP_3-receptor is identified in the *P. falciparum* although pharmacological pieces of evidence are encouraging. Further, we found the direct link of PKG mediated IP_3-induced calcium rise when the parasites were treated with the IP_3 receptor antagonist 2-ABP, where zaprinast was unable to elicit calcium flux. It will be interesting to understand the complex cross-talk of other messengers such as cAMP that also plays a crucial role in regulating the calcium level to control microneme secretion during parasite invasion. Identifying the trigger of PKG to activate the downstream

calcium efflux may likely be proven a novel therapeutic candidate considering the divergence from their mammalian counterpart.

P572/B586

An Autocrine Negative Feedback Loop Inhibits *D. Discoideum* Proliferation through a Plc/ip3/ca^{2+} Pathway.

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Very little is understood about how tissues can regulate their size. In one possible mechanism, cells in a tissue secrete a factor that inhibits their proliferation, and as the tissue gets bigger, the concentration of the factor increases. If the factor inhibits proliferation at some concentration, the resulting negative feedback loop will limit tissue size. Despite evidence for such factors, they have in general not been identified and thus their signal transduction pathways are unknown. We previously found that *Dictyostelium discoideum* accumulates extracellular polyphosphate to inhibit its proliferation. Polyphosphate inhibits the proliferation of *D. discoideum*, and this requires the G protein coupled receptor GrlD and the small GTPase RasC. To elucidate additional signaling components in the polyphosphate pathway, we screened 47 available signal transduction pathway mutants for insensitivity to polyphosphate. We found that cells lacking the G protein components Gβ and Gα3, the Ras guanine nucleotide exchange factor GefA, Phosphatase and tensin homolog (PTEN), Phospholipase C (PLC, which produces IP3 from PIP2), Inositol 1,4,5-trisphosphate receptor-like protein a (IplA, which acts as a calcium channel activated by IP3), Polyphosphate kinase 1(Ppk1), Growth-differentiation transition family members 1 and 2, Protein kinase A, or PiaA (cytosolic regulator of adenylate cyclase) showed a significantly reduced sensitivity to polyphosphate induced proliferation inhibition. In support of PLC being in the pathway, polyphosphate upregulates IP3, and this requires GrlD and PLC, and in support of IplA being in the pathway, polyphosphate upregulates cytosolic Ca^{2+}, and this requires GrlD, PLC, Ppk1 and IplA. These data suggest that polyphosphate inhibits the proliferation of *D. discoideum* through a PLC/IP3/Ca^{2+} pathway.

P574/B588

Elevated Phosphate Induces Extensive Cellular Injuries.

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Phosphate (PO_4) is an essential nutrient for human body health. It maintains and modulates normal cell functions. Insufficient intake of dietary phosphate may lead to an impaired bone development, whereas phosphate overload may cause cardiovascular calcification and premature ageing. In *vivo*, organic phosphate compounds are metabolized to inorganic phosphate in the form of dihydrogen phosphate (H_2PO_4) and monohydrogen phosphate (HPO_4). Our study focuses on inorganic phosphate's (Pi) extraskeletal effects. Evidence has shown that abnormal high level of Pi could promote cell growth and proliferation, indicating its potential role in carcinogenesis. In cancers, an increase of Pi is believed to be associated with cancer metastasis. Conversely, some studies have demonstrated that excessive Pi can kill cells and cause massive tissue damage. Most of these reported studies are confined to the phenotype description. The underlying molecular mechanisms remain unclear. Here we applied an increased amount of Pi (H_2PO_4) to mammalian cells for different durations. Cell viability/proliferation was tested by XTT assay. Furthermore, Pi perturbed cell signaling networks were explored by Western

blot analysis. We found that Pi triggers diverse cellular responses in a dose-dependent manner. At moderately higher (1mM-10mM) than physiological level, extracellular Pi could promote cell growth and survival by activating PI3K/AKT and MAPK signaling pathways. By introducing additional Pi (20-40mM), we observed significant cell injury. It was caused by interweaved Pi related biological events, including activation of MAPK signaling, ER stress, autophagy, and programmed cell death. The corresponding inhibitors or enhancers for each event were adopted to study the connections and dependency among the pathways. Beyond that, we found excessive Pi could enhance epithelial-mesenchymal transition (EMT) by inducing the EMT markers (such as increase level of phosphorylated Smad2 and Snail1, decreased expression of ZO-1) in cancer cells. This suggests the elevated extracellular Pi could promote tumor progression. Taken together, excessive extracellular phosphate causes a broad spectrum of toxicity in *vitro* by perturbing complicated signaling networks that control the cell growth, cell death, ER stress, autophagy, and cell mobility. Our results form the basis of further studies on other cell-based and mice models to deepen the understanding of phosphate induced cellular responses, and to provide therapeutic clues to minimize phosphate toxicity-associated clinical complications.

P573/B587

New Pathways That Regulate Myosin Light Chain Kinase and Cellular Force Development.

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Reversible phosphorylation of myosin light chain governs cellular force development. The binding affinity of Ca^{2+} to calmodulin defines Ca^{2+} sensitivity of the force development. In addition, the Ca^{2+} sensitivity is also reported to fluctuate in response to stimuli and contribute to the regulation of myosin light chain phosphorylation. Here, we found evidence that, in phasic smooth muscle tissues, such as intestines and urinary bladder, the Ca^{2+}-sensitivity of myosin light chain kinase (MLCK) declines after reaching at the peak force. Longitudinal smooth muscle tissues from mouse ileum were set to force transducers and subjected to partial permeabilization using beta-escin. The permeabilized tissues were relaxed in the solution with 1 mM EGTA and then stimulated with a sub maximum [Ca^{2+}] of pCa6.0 (1.0 μM). Upon the stimulation, the contraction and myosin light chain phosphorylation peaked within 30 sec and then declined to 50% of maximum level at 2 min after stimulation. The reduction in the myosin light chain phosphorylation at the clamped [Ca^{2+}] indicates the Ca^{2+}-induced Ca^{2+}-desensitization. MLCK activity in the permeabilized tissues increased by 30 sec, and then declined at 3 min after stimulation, whereas MLCP activity was unchanged at any time points in the tissue. Thus, the fluctuation in MLCK activity at clumped [Ca^{2+}] is likely a cause of the Ca^{2+}-induced Ca^{2+}-desensitization. The Ca^{2+}-induced MLCK desensitization was associated with phosphorylation at the calmodulin binding domain of the kinase. Data of pharmacological experiments using kinase antagonists suggest that unknown pathway(s) but not CaMKII, CaMKIV or AMPK are responsible for the temporal MLCK inactivation. In addition, pretreatment of the tissues with rubratoxin a potentiated the MLCK inactivation suggesting an involvement of PP2A. Novel pathways for reversible MLCK phosphorylation may define the force development in phasic smooth muscles. This work is supported by funds from Imabari city and Ehime Prefecture.

P575/B589

Ras and Cell Migration: a Rsky Signal to Myosin Phosphatase.

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Cell migration is essential to embryonic development, wound healing, and cancer cell dissemination. Cells move via leading-edge protrusion, substrate adhesion, and retraction of the cell's rear. The molecular mechanisms by which extracellular cues impinge the actomyosin cytoskeleton to control these motility mechanics are poorly understood. The growth factor-responsive and oncogenically activated protein extracellular signal-regulated kinase (ERK) promotes motility by signaling in actin polymerization-mediated edge protrusion. It has long been known that ERK can signal to activate myosin II. While a direct signal to myosin light chain kinase (MYLK) has been proposed, optimal and conserved ERK phospho-motifs are not found in MYLK. We sought to uncover the signaling mechanism by which ERK modulates myosin during cell migration. Using a combination of biochemistry and live-cell imaging, we show that ERK signals to the contractile machinery through its substrate, p90 ribosomal S6 kinase (RSK). We probed the signaling and migration dynamics of multiple mammalian cell lines and found that RSK phosphorylates myosin phosphatase-targeting subunit 1 (MYPT1) at Ser-507, which promotes an interaction of Rho kinase (ROCK) with MYPT1 and inhibits myosin targeting. We find that by inhibiting the myosin phosphatase, ERK and RSK promote myosin II-mediated tension for lamella expansion and optimal edge dynamics for cell migration. These findings suggest that ERK activity can coordinately amplify both protrusive and contractile forces for optimal cell motility.

P576/B590

Dsk1 Protein Kinase Plays a Role in Cellular Response to Stress.

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mRNA metabolic processes including pre-mRNA processing, and mature mRNA export, localization, and degradation constitute an integral aspect of gene expression and regulation. SR (serine/arginine-rich) protein kinases have a vital role in gene expression through the regulation of pre-mRNA splicing factors. The two SR protein kinases, Dis1-suppressing protein kinase (Dsk1) and Kinase in the Clk/Sty family1 (Kic1), are the *Schizosaccharomyces pombe* functional orthologs of human SRPK1 and mouse Clk1/Sty1, respectively. Prior research has demonstrated the importance of Dsk1 and Kic1 in mRNA splicing. Furthermore, nuclear retention of intron-less mRNA in Dsk1- and Kic1-null cells suggest these protein kinases also have a role in mRNA nuclear export, discrete from their role in splicing. Additionally, our lab had previously co-purified Dsk1 with major poly(A) binding protein (Pabp), an mRNA export protein that shuttles mRNA from the nucleus to the cytoplasm. We hypothesize that Dsk1 and Kic1 act on Pabp to facilitate mature mRNA export. PCR-based in *vivo* gene targeting was used to GFP tag Pabp and the related Poly(A)-binding protein 2 (Pab2) at their genomic loci in Δ*dsk1* and Δ*kic1* background strains of *S. pombe*, as well as in the two control strains: wild-type and Δ*srp1. The* effect of Dsk1 and Kic1 kinases on PabpGFP and/or Pab2GFP localization was visualized using fluorescent microscopy. We found that higher temperature promotes the formation of Pabp containing cytoplasmic foci, or stress granules, in the cells lacking Dsk1 kinase, but not in Δ*kic1* and Δ*srp1* mutants when compared to the wild-type cells. It has been posited that these stress granules are evolutionarily conserved structures, formed as a result of stress-induced polysome disassembly. The results suggest that Dsk1 is involved in *S. pombe* response to stress. It will be interesting to determine if Dsk1 or Kic1 alters the level of Pabp phosphorylation. As

Dsk1 and Kic1 are highly conserved in eukaryotes, our findings in *S. pombe* may elucidate the role of SR protein kinases in the regulation of mRNA metabolic processes in other complex eukaryotes, including humans.

P577/B591

The DSP Domain of MK-STYX in Decreasing Stress Granules.

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The DSP domain of MK-STYX in Decreases Stress Granules Fei (Krystal) Jiang and Shantá D. Hinton Department of Biology, Integrated Science Center, William and Mary, Williamsburg, VA 23187 MK-STYX [MAPK (mitogen activated protein kinase) phosphoserine/threonine/tyrosine binding protein] is a pseudophosphatase. Due to point mutations in the HC(X5)R catalytic active site signature motif, MK-STYX (FSTQGISR) can only bind, but cannot dephosphorylate phosphorylated residues. This interaction of MK-STYX allows it to play important roles in various cellular pathways including the stress response pathway. MK-STYX decreases stress granules (SG) independently of serine 149 phosphorylation of G3BP-1 (stress granule nucleator). Stress granules are cytoplasmic aggregates of untranslated mRNA and proteins formed in response to stressors. The aim of this study is to determine which domain of MK-STYX decreases stress granules. MK-STYX has two domains: a dual specificity phosphatase domain (DSP) and a rhodanese/cdc 25 phosphatase homology domain. In our study, HEK-293 cells cotransfected with G3BP-GFP and mCherry-DSP, mCherry-MK-STYX-CH2, or mChrerry. Cells expressing G3BP-GFP and mCherry-MK-STYX-DSP exhibit fewer SG than the control (G3BP-GFP and mCherry. Whereas, cells cotransfected with G3BP-GFP and mCherry-CH2 have similar levels of SG compared to the control, indicating that the DSP domain of MK-STYX is responsible for decreasing the number of stress granules. Since the “active site” lies within the DSP domain, the study suggests that MK-STYX decreases SG by binding, and perhaps blocking dephosphorylation of certain proteins. Future studies will focus on characterizing (size and exact number of these SG, as well as the interactors of MK-STYX-DSP.

P578/B592

Uhmk1 Knockdown Impacts the Expression of Epithelial-Mesenchymal Transition Genes in NIH3T3 Cells.

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UHMK1 (U2AF Homology Motif Kinase 1, also known as KIS) is a kinase that interacts with and phosphorylates the splicing factors SF1 and SF3B1. These interactions point towards an important role of UHMK1 in RNA metabolism. It is known from the literature that UHMK1 participates in proliferation, migration, membrane trafficking, RNA local translation in neurons and differentiation. Our hypothesis is that UHMK1 could contribute to these and other cellular processes by regulating alternative splicing, gene expression and phosphorylation of the involved target proteins. In order to test Uhmk1 impact on gene expression, NIH3T3 cells were retrovirally-transduced to express Uhmk1 and Uhmk1-shRNA sequences. The Uhmk1-overexpressed and -knockdown cells were submitted to RNA-seq (adapted mcSCRB-seq) and subsequent differential expression analysis was performed by edgeR/limma package. A total of 32 genes was identified as differentially expressed in Uhmk1 knockdown (p-value <0.05). Among them, 17 genes were upregulated and 15 genes were downregulated, with a log2 fold change

ranging from 0.17 to 0.72 and 0.18 to 1.66, respectively. Uhmk1 was found to be downregulated, confirming the shRNA efficiency. Research in literature showed that the human homologs of many of these genes, such as OAZ1 (Ornithine Decarboxylase antizyme 1), PIMREG (PICALM-Interacting Mitotic Regulator), CDCA4 (Cell Division Cycle Associated 4), LOX (Lysyl Oxidase), MEMO1 (Mediator of cell Motility 1), TPT1 (Tumor Protein, Translationally-Controlled 1), MAP3K3 (Mitogen-Activated Protein Kinase Kinase Kinase 3) and PRRX1 (Paired Related Homeobox 1) are differentially expressed in tumors, and related to tumor progression and aggressiveness, proliferation, Epithelial-Mesenchymal Transition (EMT) and metastasis. Gene Set Enrichment an alysis (GSEA) showed EMT, receptor complex, protein complex disassembly and phospholipid binding among the most interesting pathways with a positive enrichment ($p<0.05$). Furthermore, phosphoproteome analysis was carried out, aiming to investigate the phosphorylated proteins under Uhmk1 regulation in a Uhmk1 overexpression and knockdown context. Preliminary data showed elevated phosphorylation of Sf1 at Serine 56 localized in the SPSP domain (D3YVH4_MOUSE, UniProt) in Uhmk1 knockdown. Splicing reporter assays and invasion experiments are ongoing to validate our findings. Based on our preliminary data, we suggest that UHMK1 could have a role in EMT by controlling the splicing of related genes.

P579/B593

Swi6 Localization to Heterochromatin Is Dependent on Dsk1 and Kic1 SR-protein Kinases.

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The Serine-Threonine (SR)-Protein kinases, namely, Kinase in the Clk/Sty family1 (Kic1) and Dis1-suppressing protein kinase (Dsk1) are important regulators of cellular pathways; such as cellular viability, differentiation and various aspects of development. The *Schizosaccharomyces pombe* Swi6 (human ortholog HP1) is a transcriptional heterochromatin binding protein that is recruited to heterochromatic regions, possibly as a result of phosphorylation by the protein kinases Kic1 and Dsk1. We hypothesize that phosphorylation of Swi6 by Dsk1 and/or Kic1, is responsible for Swi6 recruitment to heterochromatic regions in *S. pombe* cells. In this study, we investigated the change in localization of Swi6, in the protein kinase *Kic1 and Dsk1* deletion mutant strains. Using the technique of PCR-based GFP-tagging, Swi6 was endogenously tagged and Swi6-GFP localization was determined through fluorescence microscopy. Swi6 was visualized through examination of condensed dotted fluorescing structures within the nucleus, which potentially correspond to the heavily heterochromatic regions of DNA in telomeres and centromeres. In Wild Type (WT) cells, Swi6 is localized to the nucleus. It was found that upon deletion of the protein kinases Kic1 and Dsk1, distinct cytoplasmic Swi6-GFP localization was observed in both strains, which was not detected in wild-type *S. pombe* strains. The incubation temperature was varied between data collections at 30 and 36 degrees Celsius. In particular, the Kic1 36ºC cells had the highest amount of cytoplasmic Swi6-GFP localization, and similar levels of cytoplasmic localization was seen in the Kic1 30ºC strain, as well as the Dsk1 30ºC and Dsk1 36ºC strains. We are interested in further investigating the Swi6 phosphorylation status in these different genetic backgrounds through the immunoprecipitation of Swi6 followed by a Western blot analysis of the cellular extracts. The specific model of Swi6 phosphorylation can be determined through in *vitro* analysis of the purified proteins Kic1, Dsk1, and Swi6. Also, genetic mutagenesis of possible phosphorylation sites on Swi6 may be used to determine the specific locus of phosphorylation responsible for Swi6 recruitment to heterochromatin.

P580/B594

Systematic Mapping of the Human Calcineurin Signaling Network Uncovers a Novel Role for Calcineurin at the Nuclear Pore Complex.

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Protein phosphatases play essential roles in cell signaling; however, systems-level understanding of phosphatases is lacking due to challenges associated with identifying their substrates and regulators. Calcineurin (CN) is the conserved Ca^{2+}/calmodulin-activated protein phosphatase and target of the widely prescribed immunosuppressant drugs, FK506 and Cyclosporin A. CN is ubiquitously expressed and plays critical roles in the immune, nervous, skeletal and cardiovascular systems, as well as during development. CN utilizes conserved docking surfaces to recognize substrates via Short Linear Motifs (SLiMs) termed PxIxIT and LxVP, which occur preferentially in intrinsically disordered domains and are challenging to identify due to sequence degeneracy and low affinity for CN. We applied novel experimental and computational approaches to systematically identify CN-binding SLiMs in the human proteome and establish the human CN signaling network. In addition to significantly expanding our understanding of CN biology in human cells, these studies identified a novel and conserved role for CN at the nuclear pore - a structure where Ca^{2+} signaling is largely uncharacterized. Through our multi-pronged approach, we identified CN-binding SLiMs on multiple nucleoporins (Nups), specifically those involved in nuclear transport. Here, we show that CN binds to conserved PxIxIT motifs in Nup153, Nup50 and TPR - all three components of the nuclear basket structure. Furthermore, proximity ligation assays (PLAs) in HeLa cells demonstrate robust interaction between CN and nuclear basket components in *vivo*. Building upon published studies showing that ERK phosphorylates key transport Nups during viral infection, oxidative stress, and in various cancer cell lines, we show that CN opposes ERK phosphosites on multiple Nups in *vitro and* in *vivo*. Furthermore, we show that inhibition of CN in HeLa cells leads to the reduced rate and accumulation of an established nuclear transport reporter. Altogether, these studies suggest that CN positively regulates nuclear transport by opposing transport-inhibitory ERK phosphosites on key transport Nups. Consistent with our interest in this Ca^{2+}-activated enzyme, our future studies will focus on identifying cellular conditions that influence Ca^{2+}dynamics at the NPC.

P581/B595

Conserved Ndr Kinase Orb6 Regulates Cell Growth and Chronological Lifespan in Response to Environmental Stress.

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Adaptation to the nutritional environment is critical for all cells. RAS GTPase is a highly conserved GTP binding protein with crucial functions for cell growth and differentiation in response to environmental conditions. Inappropriate activation of human RAS GTPase has a causal role in cancer, congenital malformations, and neurodevelopmental disorders. We previously found that the conserved NDR kinase Orb6 regulates the mRNA and protein levels of Ras1 guanine nucleotide exchange factor (GEF) Efc25, by

controlling the coalescence of the mRNA-binding protein Sts5 into ribonucleoprotein (RNP) granules (*Nuñez et al., Elife, 2016*). However, it is still unclear whether Orb6-Sts5 axis regulates RAS GTPase activity in response to the nutritional environment. Further, the molecular mechanisms by which Orb6 kinase controls Sts5 RNP coalescence are not characterized. Fission yeast *Schizosaccharomyces pombe*serves as a powerful model organism to study signaling pathways regulating nutrient sensing, cell growth, and morphogenesis due to its well-defined cell shape and growth pattern under nutrient variations. Using a phospho-specific antibody and an analog-sensitive *orb6-as2*strain, we can quantify the Orb6 kinase activity and the effect of Orb6 kinase inhibition. Here, we describe a novel mechanism tethering RAS GTPase to nutrient availability in fission yeast. The conserved NDR kinase Orb6 responds to nutritional cues and regulates Ras1 GTPase activity. Under nutrient-rich condition, Orb6 kinase increases the protein levels of a Ras1 GTPase activator, the guanine nucleotide exchange factor Efc25, by phosphorylating Serine 86 of Sts5, a protein bound to *efc25*mRNA. Under nutrient-depleted condition, Orb6 kinase activity decreases, leading to Sts5 RNP coalescence and decrease in Ras1 activity. By manipulating the extent of Orb6-mediated Sts5 coalescence into RNP granules, we can modulate Efc25 protein levels, Ras1 GTPase activity and as a result, cell growth and cell survival. The Orb6-Sts5-Ras1 regulatory axis plays a crucial role in promoting cell adaptation, balancing the opposing demands of promoting cell growth or extending chronological lifespan. Our findings highlight the conserved NDR kinase as a crucial enzyme in modulating cell growth and cell survival under favorable or adverse conditions, and in regulating cell adaptation and general stress response.

P582/B596

Effects of Polysubstituted Pyrroles on Pro-inflammatory Signaling in RAW264.7 Macrophages.

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Inflammation protects against infection and restores the body to homeostasis after cellular and tissue damage from injury or metabolic stress. Complex signaling pathways are necessary for the activation of pro-inflammatory signaling in macrophages, and dysregulation of these pathways can contribute to disease states linked to chronic inflammation making them attractive targets for therapeutics. Our laboratory explores the potential of a class of polysubstituted pyrrole compounds that are known microtubule depolymerizers to regulate the inflammatory response in macrophages. Recent work from our laboratory demonstrated that JG-03-14 (3,5-dibromo-4-(3,4-dimethoxyphenyl)-1H-pyrrole-2-carboxylic_acid ethyl ester) and its refined analog, NT-07-16, which possesses an additional methoxy group at the 2-position of the phenyl ring, decrease the production of pro-inflammatory cytokines in lipopolysaccharide (LPS)-activated RAW264.7 macrophages. The reduction in pro-inflammatory mediators appears to be due, at least in part, to a decrease in the nuclear translocation of NFκB, a critical transcription factor for pro-inflammatory signaling. Additionally, these results are supported since microtubule dynamics may be involved in NFκB nuclear translocation. Here we expand the scope of our previous work by exploring the effects of a potentially more potent stereoisomer mix of the NT-07-16 analogue, NT-07-45 on other important pro-inflammatory signaling pathways in macrophages. Although the role of the microtubule network on inflammatory signaling has not been well-studied, our results demonstrate a decrease in the production of the antiviral and pro-inflammatory cytokine interferon-beta (IFNβ) after macrophages are exposed to NT-07-45. Also, results suggest that when macrophages are exposed to NT-07-45 prior to LPS activation the phosphorylation required for the activation of the transcription factor IRF3, which is responsible for initiating the production of IFNβ, is

altered. Altering the phosphorylation of IRF3 could affect the transcription factor entering the nucleus to induce IFNβ production. Our work has also found alterations in the phosphorylation of MAP-Kinases, which is required for their activation, in macrophages that have been exposed to NT-07-45. Therefore, these findings suggest that the decrease in the production of pro-inflammatory mediators observed in macrophages exposed to these polysubstituted pyrroles may be due to the inability of multiple transcription factors involved in the inflammatory response to efficiently translocate into the nucleus. While much is still to be learned about the anti-inflammatory mechanisms of these compounds it appears that the effects may go beyond their microtubule-depolymerizing activity.

P583/B597

Making New Connection between TOR, Autophagy, and Metabolism.

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Alternative mRNA processing, including alternative splicing (AS) and polyadenylation (APA), generates transcripts with different coding and UTR regions, a mechanism that regulates RNA stability and localization, protein-protein interactions, subcellular localization, and protein expression. Dysregulation of AS and APA have been linked to numerous cancers and diseases. Here, we show that the TOR signaling pathway regulates phosphorylation of the Cleavage and Polyadenylation (CPA) complex to induce AS and APA events in the Drosophila larval fat body during nutrient deprivation. CDK8 and DOA kinases are identified as links between TOR and the CPA complex. CDK8 and DOA physically interact with and directly phosphorylate CPSF6, a key component of the CPA complex, to regulate its cellular localization and RNA-binding ability. Significantly, depletion of CDK8, DOA, and the CPA complex compromises autophagy and promotes protein, energy, and lipid metabolism during starvation. These findings elucidate a regulatory mechanism linking between the TOR signaling pathway, RNA processing and metabolism.

P584/B598

A Tug-of-war Mechanism Drives the Allosteric Activation of Protein Kinase A.

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Protein Kinase a (PKA) is an important regulatory enzyme in many signal transduction pathways. This study focuses on the mechanisms of allosteric communication between the PKA regulatory and catalytic subunits. The regulatory subunit establishes two sets of interactions with the catalytic subunit to form an inactive holoenzyme: 1) via the inhibitory sequence that docks into the active site of the catalytic subunit and is stabilized by ATP and two Mg^{2+} ions, and 2) via surface contacts between two cyclic nucleotide binding (CNB-A and CNB-B) domains. We use optical tweezers to investigate the crosstalk between the inhibitory sequence, ATP and Mg^{2+} with the surface contacts established between the CNB domains and the catalytic subunit. When ATP and Mg^{2+} are in saturation, we find that the CNB domains bound to the catalytic subunit are in equilibrium between two conformational states, a predominant conformation in which the CNB domains unfold independently of each other and at high force, and a minor conformation wherein the CNB domains unfold near-simultaneously or cooperatively and at low

force. In the absence of ATP or Mg^{2+}, a redistribution of the PKA ensemble occurs where the CNB domains only unfold cooperatively and at low force. Moreover, we investigate how the CNB-B domain controls the interaction of the CNB-A domain with the catalytic subunit. We show that the truncated CNB-A domain has a stronger interaction with the catalytic subunit compared to the CNB-A domain as part of the regulatory subunit. This result indicates that the CNB-B domain weakens the interaction between the CNB-A domain and the catalytic subunit. Altogether, this study portrays a PKA allosteric activation mechanism in which the CNB-A domain experiences a tug-of-war due to stabilizing effects conferred by the inhibitory sequence and ATP-Mg^{2+}, and destabilizing effects due to the presence of the CNB-B domain.

P585/B599

Dissecting Ligand-induced Domain Communication in Protein Kinase A.

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cAMP-dependent protein kinase (PKA) is a biomedically important protein that regulates diverse cellular signaling pathways via allosteric regulation. PKA consists of the catalytic subunit that performs phosphorylation on protein substrates and the regulatory subunit that modulates the catalytic subunit's activity. The regulatory subunit has two cyclic nucleotide-binding (CNB-A/B) domains, where the binding of cAMP initiates the propagation of signals from one domain to the other and finally releases the catalytic subunit. How the protein's communication is modulated by ligand binding remains poorly understood. Here, we used isothermal titration calorimetry and optical tweezers to investigate how ligand binding affects one domain in the presence of the other. We find that CNB-A domain has a lower cAMP affinity than CNB-B domain. The binding affinity of both CNB domains increases when the other domain is present. This result highlights the influence of one CNB domain to the other is bidirectional and asymmetric. Additionally, we investigate W260 from CNB-B that serves as the capping residue on cAMP in CNB-A to probe the driving forces behind interdomain communication. The mutation W260A exhibits a decoupling effect between the CNB domains and destabilizes the conformation integrity in the bound state. This suggests W260 has important contributions to the CNB domains' ligand-binding cooperativity and structural stability. This study allows us to dissect how ligands induce domain communications in allosteric proteins. Moreover, this study enabled the identification and characterization of aberrant allosteric events associated with mutations observed in the disease state.

P586/B600

Chemical Genetic Approaches to Choanoflagellate Tyrosine Kinase Signaling.

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Although all organisms use signal transduction to respond to external stimuli, the rise of multicellularity necessitated the evolution of signaling pathways to coordinate actions of individual cells into a singular response. Phosphorylation, a molecular event mediated by kinases that transfer the terminal phosphate of ATP onto their substrates, is an essential signaling mechanism in animals. Tyrosine kinase (TK) signaling, characterized by reversible phosphorylation on tyrosine residues by TKs and phosphatases, has profound effects on cell growth, proliferation, and migration. Although kinase signaling is expanded in all multicellular lineages, TK signaling originated in holozoans. The identification of TK signaling in

choanoflagellates, the closest living relatives of animals, suggests a role for intracellular signaling that predated the evolution of obligate multicellularity in the animal stem lineage. Since many choanoflagellates form colonies, their TKs may also regulate choanoflagellate life history transitions. The choanoflagellate *Salpingoeca rosetta* differentiates into multiple solitary and colonial forms during its dynamic life history. Like other choanoflagellates, *S. rosetta* possesses an expanded TK signaling repertoire. Advances in TK inhibitor profiling and phosphoproteomics provide complementary approaches for identifying and elucidating how TK signaling influences choanoflagellate development. We are exploring the role of TK signaling in *S. rosetta* by conducting high-throughput phenotypic screens with structurally diverse kinase inhibitors and characterizing individual *S. rosetta* TKs with functional probes. In combination with recently developed reverse genetic tools to illuminate choanoflagellate cell biology, we aim to elucidate if tyrosine phosphosignaling pathways regulate *S. rosetta* life history. Because TK signaling is conserved between choanoflagellates and animals, insights into core signaling pathways in choanoflagellates can inform our understanding of modern animal development, physiology and disease.

P587/B601

A Context Dependent Requirement for Fig4 Catalytic Function in Yeast.

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PI lipid phosphatases modulate signaling cascades through localized dephosphorylation of specific phosphoinositides (PI) on subcellular membranes. In addition, a number of these phosphatases regulate cellular processes through lipid phosphatase independent functions. Notably, Fig4, a PI 5-phosphatase proposed to dephosphorylate PI3,5P_2, has been shown to rescue neonatal defects in a Fig4 knock-out mouse suggesting that Fig4 plays important cellular roles independent of its catalytic activity. Fig4 activates its opposing kinase, Fab1, to facilitate dynamic elevation of PI3,5P_2 through association with the Fab1-Vac14-Fig4 complex; catalytically impaired Fig4 is proposed to effectively fulfill this key function. Nevertheless, mice expressing catalytically impaired Fig4 as the only copy of Fig4 display late on-set neurological abnormalities and have a reduced life span indicating that Fig4 catalytic activity is not dispensable for long-term homeostasis. Fig4 is not strictly required for growth in *Saccharomyces cerevisiae* (*S. cerevisiae*), but we show here that Fig4 confers a growth advantage to yeast cells grown on rapamycin. Strikingly, catalytically impaired Fig4 is toxic to yeast cells under these conditions, impairing growth even relative to total loss of Fig4. Toxicity of catalytically impaired Fig4 can be rescued by co-expression of wild-type Fig4 suggesting that toxicity under these conditions results from loss of catalytic function. These studies provide a new model for investigating physiological implications of loss of Fig4 catalytic activity.

P588/B602

PLK4 Phosphorylates DNA Damage Response Protein GADD45a.

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Polo-like kinase 4 (PLK4) is a serine/threonine kinase, which is best known for its role in triggering centrosome duplication. PLK4 proteins levels are thought to be maintained in a precise balance since reduced or elevated expression of PLK4 can cause numerical centrosome abnormalities resulting in aneuploidy. This can ultimately lead to malignancy. Our lab has shown that PLK4 interacts with and

phosphorylates growth arrest and DNA damage-inducible protein 45 alpha (GADD45a). GADD45a expression is induced by both p53 and BRCA1 and thus plays a role in the cells response to DNA damage in many forms such as UV and ionizing radiation (IR). GADD45a has been shown to bind to CDK1 and inhibit the interaction of the CDK1-cyclin B1 complex to induce G2/M cell cycle arrest. We have shown that heterozygous ($Plk4^{+/-}$) mouse embryonic fibroblasts (MEFs) have reduced levels of endogenous Gadd45a protein in comparison to wild-type MEFs. Our work suggests that reduced PLK4 levels correlate with GADD45a mislocalization. We have identified five putative phosphorylation sites on GADD45a targeted by PLK4 and mutated these sites to assess the effect of these phosphorylation events on GADD45a function. Flow cytometry was used to assess GADD45a activity, specifically the ability of the mutant GADD45a proteins to induce G2/M arrest. The localization of the GADD45a mutant proteins within the cell was determined as was their ability to interact with known GADD45a interacting partners such as PLK4 and NPM1. Our work in characterizing the interaction between PLK4 and GADD45a will help to better our understanding of the role that PLK4 plays in the DNA damage response and genomic fidelity.

Signaling from the PM/cytoplasm to the Nucleus

P589/B603

Crosstalk between the Hippo-YAP and Nuclear Factor-Kappa B-RELA Signaling.

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The Hippo pathway controls cell-cell interaction and organ size by regulating cell proliferation and cell death. The transcriptional factor YAP1 (yes associated protein 1) and the TEAD transcription factors are critical nuclear mediators of the Hippo pathway signaling in mammals. The RELA protein is a subunit of the nuclear factor-kappa B (NF-kappa B) transcription factors. The objective of this study was to determine whether Hippo/YAP and NF-kappa B/RELA signaling interact in living cells. Here, we have demonstrated that native YAP1/TEAD and RELA proteins biochemically and functionally interact with each other in human LNCaP and C4-2 cell lines. Our co-immunoprecipitation (co-IP), western blot (WB), and proximity ligation assay (PLA) showed that endogenous YAP/TEAD and RELA physically interact inside the cells. Our immunofluorescence assays revealed that the expression of YAP1 and RELA proteins overlapped in the cytoplasm and the nucleus. Combined treatment of cells with RANKL (receptor activator of nuclear factor-kappa B ligand) and androgen hormone enhanced YAP1 and RELA colocalization and interaction, as demonstrated by co-IP/WB experiments. Moreover, our PLA confirmed that co-treatment of cells with androgen and SDF1a (stromal cell-derived factor 1 alpha) or RANKL increased YAP1 and RELA interaction both in the cytoplasm and nucleus compared with controls. Our promoter-reporter assays showed that the knockdown of YAP1 by siRNA significantly reduced the activity of an NF-Kappa B responsive promoter-reporter gene. We also showed that controlled expression of MST1/STK4, a potent inhibitor of YAP1, attenuated the NF-Kappa B promoter reporter activity. Additionally, our unbiased bioinformatics analysis of the YAP1/TEAD and RELA chromatin immunoprecipitation data led to the identification of several genes that are potentially co-regulated by

YAP/TEAD and NF- kappa B signaling. These findings suggest that interaction between the Hippo/YAP and NF-Kappa B/RELA plays a critical role in broad cellular biology.

P590/B604

The Potential Therapeutic Mechanisms of Laminin-111 Peptide for Dry Eye Disease.

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Dry eye disease (DED) is a multifactorial disorder and often associated with chronic inflammation of the ocular surface. Integrins are heavily involved in various aspect of inflammation. In addition to modulating T cell homing by integrin αLβ2, emerging evidence suggests that integrin αvβ3 activation is critical for macrophage maintained in an inflammatory phenotype. Survival signaling induced by integrin α5β1 and αvβ3 has been discovered that is important for PMNs against extrinsic and intrinsic apoptotic signaling. Laminin-111 is known to contact and interact with integrins. Our recent animal studies shown that LDSP (a laminin-111 derived short peptide) therapeutically effective on DED and psoriasis, two inflammatory diseases. We found LDSP to be a α5β1 and αvβ3 antagonist. LDSP treatment has shown that can suppress the inflammatory phenotype (such as IL-1β) of macrophages by αvβ3-dependent manner. In addition, LDSP is able to induce PMNs apoptosis in an α5β1/αvβ3 dependent fashion. Interesting, our in vitro data reveal the corneal epithelial cells (CECs) also a LDSP target. LDSP can suppress IL-1βexpression and membrane lipid peroxidation induced by hyperosmotic stimuli. These suggest that integrin α5β1/αvβ3 may play a role for modulating inflammasome and autophagy. Our observation, for the first time, suggests that integrin α5β1/αvβ3 contributes to ocular inflammation and may a novel target for DED treatment.

P591/B605

Divergent Impacts of Nck1 and Nck2 Loss on Diabetic Podocytopathy.

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Diabetic podocytopathy (DP) is a life-threatening kidney complication that involves damage to the podocyte slit diaphragm (SD), a molecular filter of large plasma proteins. It is currently unknown if the SD signaling adaptor proteins, Nck1 and Nck2, play a role in DP pathogenesis. Nck adaptors are critical to SD function, as podocyte-specific double knockout (KO) of both Nck paralogs impairs SD formation and maintenance, resulting in severe proteinuria. Interestingly, Nck1/2 single KO mice show no overt changes in podocytes, but it is possible these mice exhibit altered susceptibility to DP. Supporting this notion, glomerular mRNA levels acquired from the NephroSeq database reveal increased *Nck1*, but decreased *Nck2* in a subset of diabetic nephropathy patients. Thus, to characterize potentially unique roles of each paralog in DP pathogenesis, we examined the degree of SD injury in diabetic Nck1 and Nck2 KO mice. Male WT, Nck1KO, and Nck2KO mice were either administered streptozotocin for diabetes induction, or citrate buffer as a respective control. Proteinuria was assessed by albumin excretion rate using metabolic cage urine collections. We found the degree of albuminuria in Nck2KO mice to be greater than diabetic WT and Nck1KO mice. Glomerular cell density, volume, and sclerosis were estimated from periodic acid-Schiff-stained kidney sections. Glomerular cell loss was observed in diabetic Nck2KOs, but not in Nck1KO or WT mice. In contrast, both glomerulosclerosis and glomerular hypertrophy were increased in diabetic WT mice, but not in Nck KOs. Finally, glomeruli were isolated and lysed to measure levels of phosphorylated and total nephrin, an important SD protein and binding partner of Nck. No alterations in phosphorylated nephrin (p1176 and pY1193) were found, but total

nephrin protein levels were higher in diabetic Nck2KOs than in diabetic WTs and Nck1KOs. In summary, these findings provide evidence of separate roles for Nck1 and Nck2 in DP pathogenesis. In agreement with the NephroSeq dataset, we have shown that a loss of Nck2, but not Nck1, is associated with increased DP susceptibility. Future studies will address whether overexpression of Nck1 may instead promote DP. Altogether, Nck adaptors should be further investigated as potential genetic factors and therapeutic targets in DP and other kidney pathologies.

P592/B606

Beta-adrenergic Receptor Signaling Reduces Pro-inflammatory Signaling and Promotes the M2 Phenotype in the Raw264.7 Macrophage Cell Line.

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Catecholamines alter macrophage function by binding to adrenergic receptors on the cell membrane. Stimulation through β_2-adrenergic receptors is known to down regulate the activity of classically activated M1 macrophages while stimulation through α-adrenergic receptors typically enhances their function. M1 macrophages initiate the pro-inflammatory response to eliminate infection while alternatively activated M2 macrophages down regulate the immune response and promote tissue repair. Our studies with the RAW264.7 murine macrophage cell line as a model for macrophage function support these findings. Macrophages were pre-treated with the selective β_2-adrenergic receptor agonist formoterol or the non-selective α-adrenergic receptor agonist clonidine prior to lipopolysaccharide (LPS) activation and analyzed for production of the proinflammatory cytokines tumor necrosis factor$_\alpha$ (TNF$_\alpha$) and interleukin-6 (IL-6) by ELISA. The results showed that formoterol decreases and clonidine increases macrophage cytokine production. The MAP-Kinase (MAPK) signaling pathway plays a role in the proinflammatory response, and our results obtained via western blotting showed that norepinephrine (NE) reduces the activation of extracellular signal-regulated kinases (ERK 1/2) and c-Jun N-terminal kinase (JNK) by decreasing the phosphorylation required to activate these MAPKs. Further studies using quantitative PCR (qPCR) showed that expression of inducible nitric oxide synthase (iNOS) mRNA was reduced while expression of arginase-1 (Arg-1) mRNA increased in macrophages pre-treated with NE prior to LPS activation. The iNOS enzyme catalyzes the production of nitric oxide, which has antimicrobial properties, and is a marker for M1 macrophages. The Arg-1 enzyme catalyzes the reaction of arginine to ornithine, a precursor to molecules participating in wound healing, and is indicative of M2 macrophages. Therefore, these findings suggest that stimulation of the adrenergic receptors by NE may also alter the phenotype of the macrophages. Additional studies using qPCR demonstrated that LPS activation decreased the expression of β_2-adrenergic receptor mRNA and increased the expression of α_{1A}- and α_2-adrenergic receptor mRNA in the macrophages, which may influence the inflammatory response in these cells. Taken together these findings suggest that exposure to NE may not only reduce the proinflammatory activity of macrophages but also induce development of the alternatively activated M2 phenotype in RAW264.7 macrophages.

P593/B607

Haptoglobin Inhibits Osteoclastogenesis through TLR4-IFN-beta Axis.

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Haptoglobin (Hp), a type of acute-phase protein, is known to have a systemic anti-inflammatory function and to modulate inflammation by directly affecting immune cells, such as T cells, dendritic cells, and macrophages. However, the effects of Hp on osteoclast differentiation are not well studied, even though osteoclast precursor cells belong to a macrophage-monocyte lineage. In this study, we found that the bone volume was reduced, and the number of osteoclasts was increased in Hp-deficient mice compared with wild type mice. Moreover, our in vitro studies showed that Hp inhibits osteoclastogenesis by reducing the protein level of c-Fos at the early phase of osteoclast differentiation. We revealed that Hp-induced suppression of c-Fos was mediated by increased IFN-b levels. Furthermore, Hp increased IFN-β level via a TLR4-dependent mechanism in bone marrow-derived macrophages. These results suggest that Hp may play a physiological role in suppressing bone loss through the Hp-TLR4-IFN-β axis in addition to inflammatory response alleviation in inflammatory-related diseases.

P594/B608

Osthole Prevents Advanced Glycation End Products-induced Renal Tubular Hypertrophy by Suppressing Translationally Controlled Tumor Protein Expression.

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Diabetic nephropathy (DN) is one of the most common diabetic complications, and advanced glycation end products (AGE) and their receptors play vital roles in pathogenesis of DN. Osthole has been widely reported to have anti-diabetic activity. Translationally controlled tumor protein (TCTP) has emerged as an important regulator of many biological processes and expressed ubiquitously in mammaliantissues. However, potential roles and molecular events for osthole and TCTP in DN remain unclear. The objective of this study was to clarify the role of TCTP in AGE-induced renal tubular hypertrophy and investigate the effect of osthole on TCTP expression. We found that raising the ambient AGE concentration causes a dose-dependent increase in TCTP synthesis in renal tubular cells. Osthole-treated and TCTP-specific siRNA-transfected cells showed significant decrease of AGE-induced TCTP protein expression. Osthole and TCTP siRNA treatments significantly attenuated AGE-induced nuclear factor-kappaB (NF-κB)/p65 activation. The decrease of inhibitor of NF-κB α (IκBα) and the increase of phosphorylated IκBα were reversed by osthole and TCTP siRNA. Moreover, the extracellular signal-regulated protein kinase (ERK) and p38 mitogen-activated protein kinase (p38 MAPK) activation were attenuated by osthole and TCTP siRNA in AGE-cultured cells. The abilities of osthole and TCTP knockdown to suppress AGE-induced cellular hypertrophy were verified by the observation that osthole and TCTP siRNA inhibited protein synthesis of RAGE, fibronectin, $p27^{Kip1}$ and α-SMA, total protein content, and cell size. Consequently, we found that osthole attenuated AGE-induced renal tubular hypertrophy via reduction of TCTP expression and suppression of the NF-κB/p65 and ERK/p38 MAPK activation. These results also showed that TCTP might be used as a unique molecular target for the treatment of DN.

P595/B609

The Transcriptional Cofactor Vgll3 Is a Novel Inducer of Epithelial-to-mesenchymal Transition.

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Epithelial-to-mesenchymal transition (EMT) is a process that converts adherent epithelial cells to motile mesenchymal cells during embryonic development and tumor progression. Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates various cellular processes, including EMT. Because TGF-β-induced EMT is involved in tumor progression, understanding of the molecular mechanisms underlying TGF-β-induced EMT is important for understanding the nature of tumors. To investigate the molecular mechanisms of TGF-β-induced EMT, we analyzed three gene expression profiles in TGF-β-stimulated cells and identified genes whose expression levels were commonly up-regulated by TGF-β stimulation. We selected several genes whose involvement in EMT was unclear and then established A549 stable lines expressing each gene. Stable expression of one of these genes, encoding VGLL3 (Vestigial-like family member 3), was found to induce morphological changes and repress expression of the epithelial marker E-cadherin. Wound-healing assays showed that stable expression of VGLL3 increased cellular mobility. These results suggested that stable expression of VGLL3 induces EMT progression in A549 cells. VGLL3 is thought to be a transcriptional co-factor for the transcription factor family TEAD (TEA-domain transcription factor). Immunoprecipitation assays showed that VGLL3 binds to TEAD1 and TEAD4, and knockdown of these TEADs repressed EMT phenotypes of VGLL3-expressing cells, suggesting that VGLL3 promotes EMT through activation of TEADs. We next analyzed the role of endogenous VGLL3 in EMT. siRNA-mediated knockdown of VGLL3 was shown to repress EMT progression upon TGF-β stimulation. High level expression of VGLL3 was observed in mesenchymal-like tumor cell lines, and knockdown of VGLL3 repressed mesenchymal-like phenotypes of these cells. These results indicated that VGLL3 is involved in EMT progression in tumor cells as well as in TGF-β-stimulated cells. Currently, analyses of molecular mechanisms of EMT progression by VGLL3 are underway.

P596/B610

PRL-1 Overexpressing Placenta-derived Mesenchymal Stem Cells Inhibit Adipogenesis in Orbital Fibroblast with Graves' Ophthalmopathy.

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Graves' ophthalmopathy (GO) is an autoimmune inflammatory disease of the orbit and is characterized by proptosis impairment of eye motility, lid retraction, de novo adipogenesis. The present medical radiotherapy results in developing malignancies. Recently, Placenta-derived mesenchymal stem cells (PD-MSCs) have unique immunomodulatory properties for tissue repair and regenerative medicine. However, PD-MSCs have a restriction on the limited self-renewal ability. Phosphatase of regenerating liver-1 (PRL-1) regulates self-renewal ability of stem cells and promotes proliferation as well as prenylated protein tyrosine phosphatases (PTPs) for inhibiting adipogenesis. Hence, the major objective is to generate and characterize PRL-1 overexpressing PD-MSCs (PRL-1(+)) using nonviral AMAXA gene delivery system and to investigate mechanisms alleviating adipogenesis by PRL-1(+) PD-MSCs in orbital fibroblast (OF) derived from GO patients. PRL-1(+) PD-MSCs were successfully generated using nonviral AMAXA gene delivery system, and analyzed their characterizations including stemness markers, differentiation, and teratoma formation. OF with GO patients induced adipogenic differentiation, and cocultured with naïve and PRL-1(+) PD-MSCs. The mRNA and protein levels of adipocyte-specific

markers, insulin-like growth factor (IGF)-binding proteins (IGFBPs), and pPI3K/AKT/mTOR pathways were confirmed by quantitative real time polymerase chain reaction (qRT-PCR) and western blotting. The characterizations of PRL-1(+) PD-MSCs were similar to naïve. OF with GO patients stimulated adipocyte differentiation was significantly decreased lipid accumulation by cocultivation with PRL-1(+) PD-MSCs compared with naïve. The mRNA and protein expression of adipogenic markers were declined in PRL-1(+) PD-MSCs (*p<0.05). The expression of pPI3K/AKT/mTOR protein in OF with GO patients was downregulated by cocultivation with PRL-1(+) PD-MSCs secreted IGFBPs. Interestingly, IGFBP2, 4, and 7 expressions through integrin alpha 4 (ITGA4) and beta 7 (ITGB7) in PRL-1+ PD-MSCs were higher than naïve and upregulated pFAK downstream factor (*p<0.05). These results indicate that secreted IGFBPs by PRL-1+ PD-MSCs inhibit adipogenesis of OF with GO patients through upregulated FAK and downregulated IGF. Taken together, these findings provide novel therapeutic approach using next-generation MSC-based cell therapy for autoimmune diseases. **Funding sources:** This research was supported by grants of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) (HI17C1050), the National Research Foundation of Korea Grant funded by the Korean Government (F19SN22T1305), Republic of Korea.

P597/B611

Initial Characterization of Hc9orf78: Regulation by Serum and Evidence of Centromere/kinetochore Localization.

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C9ORF78 is a poorly characterized protein found in diverse eukaryotes. Previous work indicated overexpression of hC9ORF78 (aka HCA59) in hepatocellular carcinoma indicating a possible involvement in growth regulatory pathways. Recently, Wang J. et al showed that Tls1, a putative fission yeast homolog of hC9ORF78, was required for telomeric heterochromatin assembly by regulating splicing of shelterin complex proteins Rap1 and Poz1 (Wang, J. et al., 2014). Consistent with this, interactome studies indicate that hC9ORF78 associates with the spliceosome (Ilagan J.O. et al., 2013). In contrast, we observed an increase in expression of TERF2IP (fission yeast homolog of Rap1) after knockdown of C9ORF78 in HeLa cells. In addition, we observed an increase in POT1 levels in contrast to no change in similar experiments in fission yeast. We hypothesize that hC9ORF78 regulates splicing differently in humans and yeast. In studies of GFP-tagged hC9ORF78 we observed a dramatic reduction in protein abundance in cells grown to confluence and/or deprived of serum growth factors. Serum stimulation induced synchronous re-expression of the protein in HeLa cells. This effect was observed with CMV-driven N-terminally GFP tagged protein, C-terminal GFP tagging of the endogenous locus, and by western blotting for the endogenous untagged protein. These data suggest C9ORF78 protein is destabilized in growth factor deprived cells. We also observe a dramatic upregulation of the shelterin component TERF2IP(Rap1 in Fission yeast) and POT1 in serum-deprived cells and are investigating to what extent this is due to hC9ORF78 regulation. Finally, we have started to characterize the subcellular localization of hC9ORF78 using GFP as well as polyclonal antibodies to the endogenous protein. hC9ORF78 localizes to nuclei in interphase, but does not appear to concentrate at speckles. Surprisingly, a subpopulation of hC9ORF78 co-localizes with ACA in mitotic cells suggesting that this protein may associate with kinetochores or centromeres. Given its association with the spliceosome, we are intrigued by the possibility that hC9ORF78 is part of splicing factors recruited at the kinetochore for processing of ncRNA during mitosis. In either case, our preliminary characterization suggests that hC9ORF78 may have multiple functions in telomere regulation, cell growth control and possible mitosis.

P598/B612

The Epithelial Innate Immune System Quantifies Microbe Associated Molecular Patterns through an Epigenetic Digital Signaling Mechanism.

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Epithelial cells perform a delicate role in the innate immune system: they must protect against biological threats without damaging the host or its commensal microbiota. Accordingly, Microbe Associated Molecular Patterns (MAMPs) must be accurately identified and quantified to execute appropriate responses. Here we use live cell imaging of innate immune signaling in mammary epithelial monolayers to study the mechanisms by which tissues and individual cells enforce appropriate quantitative responses to MAMPs and inflammatory cytokines. We conducted a comprehensive screen measuring single-cell nuclear translocation and transcriptional activation of NF-κB upon stimulation of Toll Like Receptors (TLRs) 1/2, 3, and 5, as well as IL-1R and TNFR. Our results show that epithelial cells respond to bacterial MAMPs as a population by differentially activating fractions of cells in an all-or-nothing, or digital manner. This digital signaling is maintained through epigenetic mechanisms that regulate receptor expression levels in fractions of cells. This regulation allows epithelial cells to sub-specialize within the context of a monolayer to sense identity and strength of bacterial inputs.

P599/B613

Quantitatively Defining Wnt Signaling in Colon Cells.

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WNT signaling is involved in organism development, maintenance of proliferative stem cells, and development of disease. We seek to uncover how individual cells mount a robust response to activation of the WNT pathway. Using *Xenopus laevis* oocytes, previous work in our lab shows positive feedback between two components of the destruction complex - glycogen synthase kinase 3 (GSK3) and the scaffolding protein Axin. GSK3 stabilizes Axin by preventing its degradation over time. Axin binds GSK3, targeting GSK3 for dephosphorylation by protein phosphatase 2A (PP2A), leading to its activation. Mathematical modelling using ordinary differential equations predicts a bistable response to WNT activation. We experimentally validate the model in non-malignant human colonic epithelial cells (HCECs). We activate the WNT pathway using the GSK3 inhibitor CHIR99021 (GSK3i), using nuclear localization of the WNT effector protein β-catenin as readout, measured by high throughput microscopy. We measured a feature of a bistable system called hysteresis. HCECs respond to GSK3i in a hysteretic manner, showing about 2-fold decrease in EC_{50} of cells that were pre-activated ($EC_{50} \pm$ SEM = 1.7 μM $\pm$ 0.4) with a high dose of GSK3i (10 μM) compared to naïve group (DMSO control; $EC_{50} \pm$ SEM = 3.3 μM $\pm$ 0.9). Our data show that positive feedback between GSK3 and Axin lead to a hysteretic and therefore, bistable response to activation of the WNT pathway through GSK3 inhibition. Future studies will determine if HCECs that possess tumor suppressor and oncogenic mutations lose their bistable response to GSK3i.

P600/B614

Spi-C Enhances Osteoclast Differentiation by RANKL Stimulation.

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SPI-C is a SPI-group ETS transcription factor expressed temporarily during B-cell development and in macrophages. However, it has so far been little studied on the involvement of SPI-C with osteoclastogenesis. Osteoclasts are multinucleated cells derived from hematopoietic progenitors of the bone marrow-derived monocyte/macrophages. We present here that SPI-C is a novel positive regulator of RANKL-induced osteoclast differentiation. Spi-C is up-regulated during osteoclast differentiation, and the knockdown of Spi-C effectively decreased the RANKL-induced osteoclast marker gene expression as well as resulted in a remarkable decrease of the number of TRAP-positive multinucleated cells. Spi-C overexpression significantly enhanced osteoclast formation by RANKL and the knockdown of Spi-C blocked the activation of MAP kinases and IκBα by RANKL. RANKL-mediated Spi-C expression was JNK-dependent, and the translocation into nucleus of Spi-C was suppressed by the inhibitors of p38 MAP kinase and PI3K. BMMs pretreated with these inhibitors failed to differentiate into osteoclasts. Collectively, these results suggest that Spi-C plays a critical role in RANKL-induced osteoclast differentiation. *This work was supported by NRF-2016R1D1A1B01012205.

P601/B615

Lgr-independent Potentiation of Wnt Signaling by R-spondins: Mechanism and Functions in Development.

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The WNT signaling pathway encodes positional information in animals, orchestrates patterning and morphogenesis during embryonic development, and promotes tissue renewal and regeneration in adults. Dysregulation of WNT signaling has been implicated in many diseases, including developmental malformations, skeletal and dental abnormalities, cardiovascular and neurodegenerative disorders, diabetes, and many types of cancer. Members of the R-spondin family of secreted growth factors have emerged as key regulators of WNT signaling strength. The R-spondin system is a vertebrate adaptation that may be related to the appearance of sophisticated stem cell compartments, which require exquisite control of WNT signaling. The four members of the family (R-spondin 1-4) can strongly potentiate responses to WNT ligands during development and in stem cells, but the mechanisms by which they transduce signals are not fully understood and the reasons why they produce different physiological effects are largely unknown. Unexpectedly, we discovered that R-spondins 2 and 3 can uniquely potentiate WNT signaling in cells lacking LGRs 4, 5 and 6, the principal R-spondin receptors. We determined the protein domains on R-spondins necessary and sufficient for this new mode of signaling, and showed that it is mediated by an alternative interaction with cell-surface heparan sulfate proteoglycans. This finding is transformative because LGRs 4-6 were thought to be required to transduce all R-spondin signals and hence determine their site of action. Indeed, these LGRs are highly expressed in many stem cell compartments whose maintenance depends on potentiation of WNT signaling by R-spondins. Instead our work shows that there are two modes of signaling by R-spondins, an LGR-dependent and an LGR-independent mode, and that different R-spondins may use distinct molecular mechanisms to transduce signals in various biological contexts. Supporting our findings, recent work from another group demonstrated that during limb development, R-spondin 2 signals through an LGR-

independent mechanism. I will present the results demonstrating our conclusions and discuss their implications in development and tissue homeostasis.

P602/B616

BMP Receptor Agonists: a Rare Find in BMP Receptor Pharmacology.

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Bone Morphogenetic Proteins (BMPs) are set of powerful morphogens which play critical roles in development of the nervous system, regulating activities such as differentiation and migration of progenitor cells, as well as formation of neural circuits. BMPs are known to play fundamental roles in many organ systems like skeletal, cardiovascular, pulmonary and gastrointestinal. They are also required for maintaining homeostasis in adult tissue like facture repair, vascular remodelling and maintaining joint integrity. Because of their ubiquitous presence in different organ systems, perturbation in BMP signaling can lead to marked developmental defects or severe pathologies later in life. BMP signaling is highly regulated by intracellular, extracellular and membrane modulators. Currently, the small molecules known to regulate BMP signaling are largely antagonists. The few compounds that have been identified as positive regulators of BMP activity, primarily act as enhancers or sensitizers. To date, no full agonist of BMP signaling has been identified. In this study, we have analysed a novel series of compounds, which share a core structure with an identified sensitizer of BMP activity (PD407824). Our results suggest that select compounds from this series of small molecules demonstrate BMP receptor agonist activity by stimulating both Smad- and PI3K-dependent signaling pathways in C2C12 cells, as well as in human pulmonary endothelial cells. The active compounds also appear to act as enhancers of BMP activity when used in combination with subthreshold concentrations of BMPs. Morevoer, we have determined that stimulation of Smad phosphorylation by our active compounds is dependent on signaling through type I BMP receptor subunits by using the type I BMP receptor inhibitor, Dorsomorphin. We are now exploring the functional similarities of these compounds to the transcriptional, inductive and chemotropic actions of BMPs by 1) determining whether these compounds upregulate target genes similar to those stimulated by BMPs, as well as, induce the differentiation of C2C12 myoblasts into osteoblasts using RT-PCR and ALP assays and 2) assessing whether these compounds promote chemotropic activity using chemotaxis and wound-healing assays. Identification of potent BMP receptor agonists would be valuable for controlling stem cell differentiation, as potential therapeutics for the treatment of BMP-related disorders, such as pulmonary arterial hypertension, or for promoting wound healing.

P603/B617

Investigating the Effects of Resveratrol on Combating Inflammation Induced by Exposure to Inhaled Pollutants.

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The goal of this research was to evaluate the anti-inflammatory effects of resveratrol on macrophage inflammation induced by woodsmoke and acrolein exposure. Resveratrol is a compound normally found in the skin of red grapes that has anti-inflammatory, antioxidant, metabolic, and cardioprotective qualities. Woodsmoke is generated from the burning of wood, whereas acrolein is an aldehyde derived from smoke in house and forest fires, cigarette smoke, cooking oil, etc. Although there is limited published research on resveratrol as an option to limit the effects of inflammation, existing research

shows that it works by regulating, activating, or deactivating various regulatory pathways and cytokine production (via the NF-kB signaling pathway). We exposed a murine RAW 264.7 macrophage cell line to lipopolysaccharide (LPS) and zymosan (two compounds that work to stimulate the innate immune response) and woodsmoke. After toxicant exposure, we used techniques including Lactate Dehydrogenase (LDH) assay, Polymerase Chain Reaction (PCR)/gel electrophoresis, and Sandwich Enzyme-Linked Immunosorbent Assay (ELISA) to assess the ability of resveratrol to inhibit inflammation. These assays tested for cellular cytotoxicity, inflammatory gene expression, protein production, etc. Thus far, our data suggest that macrophages exhibit increased levels of pro-inflammatory gene expression when exposed to LPS, zymosan, and woodsmoke alone. We also saw that when increasing concentrations of resveratrol were added, resveratrol had some inhibitory effects on woodsmoke (regarding gene expression and protein production), but not observed effect on LPS and zymosan. The downstream signaling pathways are known for LPS and zymosan, but the pathways leading to woodsmoke-induced inflammation are currently unknown. These data suggest that the signaling pathways used for LPS and zymosan are clearly different from that of woodsmoke. In the future, we wish to continue to gather data on woodsmoke signaling pathways, acrolein exposure, and other assays to look at levels of phagocytosis and cell surface protein changes.

P604/B618

Pact Facilitates Activation of the Notch Signaling Pathway by Strengthening the Association between Cbf1 and Notch Intracellular Domain.

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The protein activator of protein kinase R (PKR) (PACT) is known to play important roles in PKR regulation and microRNA biogenesis. Based on the observation that PACT is specifically expressed in the ventricular zone (VZ) at the mid-neurogenic period, we examine the role of PACT in this embryonic neural stem cell niche. Here, we provide the first evidence that PACT increases neurosphere formation, as well as expression of Notch target genes and the neural stem cell marker Sox2 in primary neural stem cells in *vitro*. Consistently, introduction of PACT into the mouse embryonic brain in *utero* increased the fraction of cells localizing to the VZ. We also show that the PACT-enhanced stemness of neural stem cells is PKR-independent. At the molecular level, PACT was revealed to physically interact with C promoter binding factor 1 (CBF1) and dramatically strengthen the association between CBF1 and Notch intracellular domain (NICD), which indicates stabilization of the Notch transcriptional coactivation complex responsible for Notch target gene expression. Taken together, our study indicates that PACT is a novel transcriptional coactivator of the Notch pathway playing a pivotal role during mammalian brain development.

P605/B619

P63 Control of Desmosomal Gene Regulation and Adhesion Is Compromised with Mtorc1 Loss of Function.

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The transcription factor p63 (isoforms TAp63 and DNp63) is essential for the formation of epidermis and other stratifying epithelia as demonstrated by the severe skin abnormality of p63-deficient mice and by the development of certain types of ectodermal dysplasia in humans as a result of p63 mutations.

However, the molecular determinants of p63 regulation and its specific role in the transcriptional program regulating epidermal fate remain poorly characterized. Patients with AEC (Ankyloblepharon, ectodermal defects, cleft lip/palate) syndrome caused by mutations in p63 exhibit severe skin fragility and blistering due to reduced desmosomal gene expression. Studies from our lab previously showed that mTORC1 is an essential driver of epidermal morphogenesis, stratification and desmosomal gene regulation, with epidermal Rheb and Rptor cKO mice exhibiting a similar phenotype as seen in AEC patients and p63-null mice. We investigated the role of p63 in mediating this phenotype, downstream of mTORC1. Mice with mTORC1 loss of function have reduced basal nuclear p63 expression in the epidermis. *Rptor* floxed keratinocytes treated with cre recombinase (*Rptor* cre) showed decreased whole cell and nuclear expression of TAp63 and DNp63 isoforms by western blotting. Over-expression of both p63 isoforms in *Rptor* cre keratinocytes rescued expression of desmosomal proteins and cellular adhesion, as seen by western blots and dispase assays respectively. In mice with mTORC1 loss of function, we previously found that Rho kinase (ROCK) signaling was constitutively activated, resulting in increased cytoskeletal tension and impaired cell-cell adhesion, and ROCK inhibition rescued desmosomal levels and adhesion. mTORC1 loss of function also resulted in marked feedback upregulation of upstream TGF-β signaling, triggering ROCK activity and its downstream effects on desmosomal gene expression. However, the transcription factors mediating these effects remain uncharacterized. A recent study reported that ROCK is a negative regulator of p63 and we found whole cell and nuclear levels of p63 to be rescued by ROCK inhibition in *Rptor* cre keratinocytes by western blotting. Whole cell and nuclear levels of p63 was also rescued by TGF-β inhibition in *Rptor* cre keratinocytes by western blotting. Increased nuclear localization of p63 was also observed in *Rptor* cre keratinocytes with TGF- β signaling inhibition. These data suggest that p63 expression and/or activity may be regulated by TGF-β signaling upstream of mTORC1 and ROCK signaling downstream of mTORC1.

P606/B620

Endosomal Regulation of DAF-16 FOXO and Insulin/IGF Signaling.

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The insulin/IGF signaling pathway negatively regulates a conserved family of FOXO transcription factors that play a major role in aging, metabolism and stress responses. Phosphorylation of FOXO by AKT kinases leads to 14-3-3 binding and sequestration of FOXO in the cytoplasm. Subsequent ubiquitination targets FOXO for degradation. However, it is not known if extranuclear FOXO is compartmentalized or just diffuse in the cytoplasm. *C. elegans* has a single FOXO, DAF-16. We investigated DAF-16::GFP subcellular localization by epifluorescence and confocal microscopy. We found that DAF-16 localizes to vesicles in the intestinal cells. The number of vesicles per cell is variable, ranging from zero to much greater than 50. This localization is partly dependent on insulin/IGF signaling and the RLE-1 E3 ubiquitin ligase. We find that DAF-16-positive vesicles correspond to a subset of RAB-5 and RAB-7 positive endosomes. In *tbc-2* mutant worms, which have increased RAB-5 activity, endomembrane localization of DAF-16 is greatly expanded at the expense of nuclear localization. We found that TBC-2 is required for the increased fat storage and extended lifespan of *daf-2 IGF receptor* mutant animals suggesting that endomembrane localization serves to sequester and or inhibit DAF-16. We are testing if TBC-2 regulates the expression of DAF-16 target genes using transcriptional reporters and qRT-PCR. These findings identify endosomes as a potential site of DAF-16 regulation and suggest that FOXO proteins could similarly be regulated on endosomes in human cells.

P607/B621

Translational Regulation of Cav2.2 by the Sigma-1 Receptor at the Dorsal Root Ganglia during Neuropathic Pain.

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Primary sensory neurons at the dorsal root ganglia (DRGs) signal for neuropathic pain, when pathophysiologic damages elicita hyperexcitability in DRG neurons. We have previously demonstrated that the blockade of the sigma-1 receptor (Sig-1R) can reverse the diminished inward Ca^{2+} current (I_{ca}) which is a known feature of nerve injury in primary sensory neurons. One of the potential Ca^{2+} channels responsible for the I_{ca} is Cav2.2. In the present study we examined the potential transcriptional and translational controls of Cav2.2 by the Sig-1R in DRG during neuropathic pain induced by spared nerve injury (SNI) and explored the underpinning molecular mechanism thereof. To this end, the Cav2.2 protein expression level, and the mRNA level were compared in DRGs obtained from skin shame (SS) control and SNI rats. Immunoblotting showed a decrease of the Cav2.2 protein expression but not its mRNA level, indicating either a protein degradation or a translational regulation. In the protein degradation test, we found that ubiquitination of Cav2.2 did not differ in wild-type or Sig-1R knockout (KO) HEK cells under the condition when proteasome is inactivated by using NG132. This suggested that the decrease of Cav2.2 protein is related to translational processes. Using the RNA-immunoprecipitation assay, we found that the eukaryotic initiation factor eIF4E can increase its binding to Cav2.2 mRNA in Sig-1R KO HEK cells when compared to wild-type HEK cells. On the other hand, it has been demonstrated that 4E-BP1 can interact with eIF4E and attenuate the eIF4E binding to the mRNA 5'-cap to decrese the initiation of the translation of the mRNA. We reasoned that the Sig-1R may regulate 4E-BP1.We found that 4E-BP1 protein and mRNA were both decreased in Sig-1R KO HEK cells and interestingly were both increased in SNI DRGs, suggesting that the Sig-1R upregulates the transcription of 4E-BP1. Further, although 4E-BP1 did not directly interact with Sig-1R in HEK cells, 4E-BP1 increased its interaction with eIF4E in SNI DRGs, blunting the initiation of mRNA translation. Taken together, our results suggest that a decreased of Cav2.2 expression after nerve injury is due to the inactivation of eIF4E which in turn is governed by the level of the 4E-BP1 seemingly controlled by the Sig-1R. However, the Sig-1R regulation on the 4E-BP1 expression needs further examination.

Dynamics of Focal Adhesions and Invadosomes

P608/B623

Analyzing the focal adhesion mechanics using novel Src and p130Cas-based FRET Biosensors.

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Focal adhesions (FAs) are dynamic adhesion structures mediating bidirectional mechanical and signaling link between ECM and intracellular actin cytoskeleton. Many FA-constituting proteins have the ability to function as mechanosensors; proteins that respond to mechanical stress by changing their conformation and transmit the mechanical stimuli to cellular response. P130Cas and more recently also Src tyrosine kinase were proposed to function as such mechanosensors in FAs. To analyze the role of Src and its main substrate in FAs p130Cas we have developed FRET-based biosensors allowing us to monitor

conformational changes and activity of Src, p130Cas phosphorylation and tension induced stretch of substrate domain of p130Cas in live cells. Based on the results obtained with the Src-FRET biosensor we proposed a new model for the role of Src and Src-dependent phosphorylation in focal adhesions. According to the model, Src is rapidly activated during focal adhesions assembly and Src-dependent tyrosine phosphorylation of focal adhesion proteins drives the assembly phase of focal adhesions. It remains stable during maturation of focal adhesions due to counteracting activity of tyrosine phosphatases. Hyperphosphorylation of the Src targets in focal adhesions, potentially due to decreased activity tyrosine phosphatases, induces focal adhesions' disassembly which is followed by gradual Src inactivation. References: 1. Koudelková L, Pataki AC, Tolde O, Pavlik V, Nobis M, Gemperle J, an derson K, Brábek J, Rosel D. Novel FRET-Based Src Biosensor Reveals Mechanisms of Src Activation and Its Dynamics in Focal Adhesions. Cell Chem Biol. 2019 Feb 21;26(2):255-268.e4. 2. Braniš J, Pataki C, Spörrer M, Gerum RC, Mainka A, Cermak V, Goldmann WH, Fabry B, Brabek J, Rosel D. The role of focal adhesion anchoring domains of CAS in mechanotransduction. Sci Rep. 2017 Apr 13;7:46233.

P609/B624

The Role of APC-mediated Actin Assembly in Microtubule Capture and Focal Adhesion Turnover.

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Focal adhesions (FA) are highly dynamic structures that assemble at the front of the cell and disassemble at the rear, allowing cells to move directionally. Focal adhesion (FA) turnover depends on microtubules and actin. Microtubule ends are captured at FAs, where they induce rapid FA disassembly. However, actin's roles are less clear. Here, we use polarization-resolved microscopy, FRAP, super-resolution microscopy, live-cell imaging, and a mutant of Adenomatous polyposis coli that is incapable of nucleating actin (APC-m4) and impairs directional cell migration (Juanes et al., 2017; *J. Cell Biology*) to investigate the role of actin assembly in FA turnover. We show that APC-mediated actin assembly is critical for maintaining normal F-actin levels, organization, and dynamics at FAs, along with proper organization of FA components, including Src and FAK Kinases, and Paxillin. Live imaging in wild type cells shows that microtubules are captured repeatedly at FAs as they mature, but once a FA reaches peak maturity, the next microtubule capture event leads to delivery of an autophagosome, triggering FA disassembly. In APC-m4 cells, microtubule capture frequency and duration are altered, and there are long delays between autophagosome delivery and FA disassembly. Thus, APC-mediated actin assembly is required for normal feedback between microtubules and FAs, and maintaining FAs in a state 'primed' for microtubule-induced turnover. In addition, we found that APC-m4 expression or silencing of the formin Dia1 each caused a ~30% reduction in total F-actin levels in cells; however, Dia1 silencing led to only modest defects in FA turnover. Thus, APC-m4 effects on FA turnover do not arise from a general loss of actin assembly in cells, but rather from a specific disruption of APC-mediated actin nucleation at FAs. In summary, this work demonstrates that APC is a *bona fide* actin nucleator in *vivo*, and that this activity of APC is crucial for regulating FA turnover underlying directed cell migration. Importantly, our results do not rule out the possibility of other actin assembly-promoting factors contributing to FA turnover, or APC coordinating actin and microtubule dynamics in other cellular locations besides FAs.

P610/B625

Limd1 Phase Separation Contributes to Cellular Mechanics and Durotaxis by Regulating Focal Adhesion Maturation Under Force.

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Cells sense and respond to extra-cellular mechanical environment through cell-matrix adhesions. Interestingly, the maturation of focal adhesions (FAs) is reciprocally force-dependent. How this mechano-chemical coordination is achieved at cellular and molecular levels remains enigmatic. Meanwhile, how biomechanical cues in the extracellular environment dictate the status of cell motility is yet to be determined. LIMD1, a member of the LIM domain proteins, localizes to the FAs and has been reported to negatively regulate the Hippo-YAP signaling pathway in response to tension. Here we identify the force sensitive recruitment of LIMD1 to the FAs. We discover that LIMD1 maintains FA dynamics and composition, and regulates cell spreading and cellular force. FAs mediate the cross-talk between the cytoskeleton and the extracellular matrix during migration. We found that the directional cell migration towards a stiffness gradient-durotaxis is hampered upon LIMD1 deficiency. Intriguingly, LIMD1 selectively recruits late but not early FA proteins through phase separation. We further discover that vinculin may act as an anchor for LIMD1 phase separation under force. Our data suggest a model in which localization of LIMD1 to the FAs, triggered by mechanical force, serves as a phase separation hub for assembling and organizing late FA proteins, allowing for effective FA maturation and efficient cellular mechano-transduction.

P611/B626

The Architecture of Talin Reveals an Autoinhibition Mechanism.

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Focal adhesions (FAs) are protein machineries essential for cell adhesion, migration and differentiation. Talin is an integrin-activating and tension-sensing FA component directly connecting integrins in the plasma membrane with the actomyosin cytoskeleton. However, its regulation is poorly understood. Here, we show a cryo-EM structure of full-length talin revealing a two-way mode of autoinhibition. The actin-binding rod domains fold into a 15-nm globular arrangement that is further interlocked by the integrin-binding FERM head. In turn, the rod domains R9 and R12 shield access of the FERM domain to integrin and the phospholipid PIP2 at the membrane. This mechanism ensures synchronous inhibition of integrin-, membrane- and cytoskeleton-binding. We also demonstrate that compacted talin reversibly unfolds to a ~60-nm string-like conformation, revealing interaction sites for vinculin and actin. Our data explains how fast switching between active and inactive conformations of talin could regulate FA turnover, a process critical for cell adhesion and signaling.

P612/B627

Regulation of PARP-1 Localization by Phosphorylation at Ser ^{372} by ERK1/2.

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The poly(ADP-ribose) polymerases-1 (PARP-1) is a highly abundant nuclear protein. Its nuclear functions in repairing DNA damages induced by various stress signals, including genotoxic and inflammatory

stresses, have been well studied. In contrast, limited studies have been done on a cytoplasmic role(s) of PARP-1. Our study focuses on the cytoplasmic role of PARP-1 during microglia activation. Using immunofluorescence microscopy and western blotting, we showed that PARP-1 is present in the cytosol of microglia cells stimulated and activated by LPS. PARP-1changes its localization between nucleus and cytoplasm and more PARP-1 is in the cytosol of activated microglia. This change of PARP-1 localization can be blocked by U0126, an ERK1/2 inhibitor. The role of ERK1/2 phosphorylation on Ser^{372} of PARP-1 in the regulation of PARP-1 activity has been reported, but it has never been linked to the regulation of PARP-1 localization. Cells transfected with GFP-$PARP\text{-}1^{S372E}$ show a high level of PARP-1 in the cytosol in the resting microglia. In contrast, GFP-$PARP\text{-}1^{S372A}$ shows almost exclusive localization in the nucleus even in activated microglia. These results suggest a novel regulatory mechanism underlying cytoplasmic localization of PARP-1 by ERK1/2. We also demonstrated a novel function of cytoplasmic PARP-1 in regulating focal adhesion (FA) disassembly. In resting microglia, co-localization between paxillin, a main component of FA, and cytoplasmic PARP-1 was observed in resting microglia. Increased cytoplasmic PARP-1level induced by LPS was accompanied by focal adhesion assembly. These results suggest that PARP-1 localization change have a regulatory effect on FA disassembly, which may further regulate cell motility upon microglia activation.

P613/B628

Development of an Invadosome Inhibitor for Cancer Cell Metastasis Suppression.

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Invadosome, a term composed of invadopodia and podosome, is an actin-rich structure featured with ECM degradation ability. Depending on different cell types, invadosomes participate in various cellular functions, including migration of immune cells, bone resorption of osteoclasts, and especially the metastasis of cancer cells. Tks5, which contains a PX domain and five SH3 domains is a critical regulator for invadosome formation. In invadosomes, Tks5 acts as a scaffold that the PX domain determines the localization of invadosomes, and the SH3 domains recruit actin polymerization proteins such as N-WASP and Grb2 to invadosomes. Previous studies have shown that overexpression of Tks5ΔPX, a truncated protein lacking PX domain, could inhibit invadosome formation; therefore, it is unclear whether any one of these five SH3 domains could inhibit invadosome formation. In this study, we are aiming to develop an invadosome inhibitor to attenuate the invasion ability of cancer cells. Surprisingly, we found that overexpression of two SH3 domains could effectively inhibit invadosome formation in NIH3T3 c-Src cells. Besides, we generated the cell-penetrating peptide fusion proteins for SH3 proteins delivery. To further analyze the effect of SH3 proteins treatment on the invadosome functions and other cellular processes such as endocytosis, we will perform the ECM degradation assay, invasion assay, and transferrin uptake with the human breast cancer cells, MDA-MB-231. We hypothesize that single SH3 domain from Tks5 could be utilized as a potent invadosome inhibitor.

P614/B629

Modular Actin Nano-architecture Enables Podosome Protrusion and Mechanosensing.

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Basement membrane transmigration during embryonal development, tissue homeostasis and tumor invasion relies on invadosomes, a collective term for invadopodia and podosomes. An adequate structural framework for this process is still missing. Here, we reveal the modular actin nano-architecture that enables podosome protrusion and mechanosensing. The podosome protrusive core contains a central branched actin module encased by a linear actin module, each harboring specific actin interactors and actin isoforms. From the core, two actin modules radiate: ventral filaments bound by vinculin and connected to the plasma membrane and dorsal interpodosomal filaments crosslinked by myosin IIA. On stiff substrates, the actin modules mediate long-range substrate exploration, associated with degradative behavior. On compliant substrates, the vinculin-bound ventral actin filaments shorten, resulting in short-range connectivity and a focally protrusive, non-degradative state. Our findings redefine podosome nanoscale architecture and reveal a paradigm for how actin modularity drives invadosome mechanosensing in cells that breach tissue boundaries.

P615/B630

Macro-based an alysis of Podosomes, Their Components and Contact by Microtubule +tips.

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Podosomes are actin-based adhesion and invasion structures in a variety of cell types, with podosome-forming cells displaying up to several hundreds of these structures. Podosome numbers, distribution and composition can be affected by experimental treatments or during regular turnover, necessitating a tool that is able to detect even subtle differences in podosomal properties. Here, we present a Fiji-based macro code termed “Podji” (“podosome analysis by Fidji”), which serves as an easy-to-use tool to characterize a variety of cellular and podosomal parameters, including area, fluorescence intensity and relative enrichment of associated proteins. This tool should be useful to gain more detailed insight into regulation, architecture and functions of podosomes. Moreover, it should be easily adaptable for the analysis of other micron-size punctate structures such as the podosome-related metalloproteinase islets, invadopodia, or focal complexes. This poster describes the work flow of the Podji macro, presents several examples of its applications, and also points out limitations, as well as adaptable features to streamline the analysis. We also present a second macro that is used to quantitatively analyse the contact of microtubule +TIPs with podosomes. Combining a tracking approach with +TIP detection in live cells, we analyse the impact of +TIP/microtubule contacts on the delivery of vesicle cargo to and accumulation at podosomes. In particular, we investigate i) the role of the ring structure as a potential docking site for MT1-MMP vesicles, and ii) the impact of actomyosin-generated forces regulated by the podosome cap on +TIP tethering at the ring, on substrates of different stiffness.

P616/B631

Investigating the Role of Specc1l Drosophila Homolog, Split Discs, in the Regulation of Non-muscle Myosin II Contractility.

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Point mutations to sperm antigen with calponin homology and coiled-coil domain 1 like (SPECC1L), have been associated with a spectrum of crainio-facial disorders including Tessier type IV clefts, Oblique facial clefts, Opitz G/BBB syndrome, and Teebi hypertelorism all of which commonly share pathologies such as cleft palate or lip, hypertelorism (wide- spaced eyes), and defects of larynx, and esophagus [2,3,4].

These phenotypes are suggestive of defects in the migration and adhesion of neural cranial crest (NCC) cells [1]. Interestingly, morpholino knock-out of the zebrafish homolog of SPECC1L led to defects in the formation of head and jaw structures, and depletion of the Drosophila homolog of SPECC1L, Split Discs (SPDS), led to defects in the formation of the proboscis and blistering in wings [1]. Collectively, these phenotypes are indicative of defects in cellular adhesion and migration. In mammalian cells, SPECC1L co-localized with both actin and microtubules, and as such was hypothesized function as a cytolinker [1,5]. When we expressed SPDS in Drosophila S2 cells we observed a localization pattern more consistent with nonmuscle myosin II (NMII). Given this potential localization we hypothesized that SPDS may affect cell migration through the regulation of NMII contractility. To test these hypotheses, we carried out a series of colocalization experiments between SPDS and NMII, including point mutations analogous to those associated with cranial-facial disorders in humans. Furthermore, we used a migratory Drosophila cell line (D25) and tracked focal adhesion dynamics. We found that the phosphorylation status of SQH had no effect on the association between SPDS and SQH, while point mutations to SPDS did. Our data indicate that SPDS does colocalize with NMII, with the point mutation Q266P showing the highest degree of colocalization while G915S showed the lowest. We also found that while depletion of SPDS by RNAi had no effect of focal adhesion assembly rates, however, the rates of focal adhesion disassembly were slowed. Furthermore, the overall rate of cell migration was also decreased following depletion of SPDS, suggesting that this delay in focal adhesion disassembly may be affecting cell migration. We conclude that SPDS localizes to NMII and that it plays a role in focal adhesion dynamics that may be mediated by this interaction.

Signaling Networks Governing Cell Migration

P617/B632

Mechanisms of Sema-5c Signaling during Collective Cell Migration in the *Drosophila* Follicular Epithelium.

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The collective migration of cells is a critical process during development, wound healing, and cancer metastasis. We study the highly coordinated collective migration of follicular epithelial cells in the *Drosophila* egg chamber. These cells utilize planar polarized signaling proteins to coordinate their migration, which is required to create the ovoid shape of the egg; follicle cells that do not migrate produce round eggs. Recent work in our lab has identified a relatively understudied transmembrane semaphorin, Sema-5c, as a novel regulator of migration. However, the molecular details of how Sema-5c regulates migration are unknown. Normal migration involves the extension of actin-rich protrusions only from the leading edge of each cell. Through live imaging, we have observed the extension of protrusions from the plasma membranes at not only leading edges, but also trailing edges in egg chambers lacking Sema-5c. We have also begun to characterize the role of a putative downstream target of Sema-5c, the small GTPase Rap1, in follicle cells during migration. Expression of a constitutively active form of Rap1 ($Rap1^{CA}$) produces moderately round eggs, which is strikingly similar to the shape of *Sema-5c* null eggs. Furthermore, we find that egg chambers expressing $Rap1^{CA}$ have slowed migration rates, suggesting that Rap1 has some function in follicle cell migration. Indeed, Rap1 is found at the basal surface of cells, where the molecular machinery controlling migration is located. Finally, we have taken advantage of the fact that egg chambers null for *Sema-5c* produce moderately round eggs that are neither fully elongated nor completely round to conduct an enhancer/suppressor screen. Previous work has shown that

removal of the receptor protein tyrosine phosphatase Lar, another regulator of migration in follicle cells, suppresses the *Sema-5c* egg shape defect. We are currently screening the left arm of the second chromosome, which contains about 20% of the *Drosophila* genome, using large deletions to tile across the entire arm. Though this screen, we aim to identify new proteins that interact with the Sema-5c signaling system and that may have roles in regulating migration.

P618/B633

Cell Confinement Reveals a Branched-actin Independent Circuit for Neutrophil Polarity.

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Migratory cells use distinct motility modes to navigate different microenvironments, but it is unclear whether these modes rely on the same core set of polarity components. Here, we address this question by dissecting the role of branched actin assembly in the neutrophil polarity program. We use CRISPR-mediated genome editing in human neutrophils to knock out either the Arp2/3 complex, the nucleator of branched actin assembly, or its key activator the WAVE complex, which promotes branched actin assembly at the leading edge of migratory cells. We find that during unconfined migration, Arp2/3 and WAVE complex are each required for the mechanical force generation that supports long-range negative feedback and restriction of protrusion growth to one location. However, each complex is dispensable for polarity and movement of confined cells, where cell-extrinsic mechanical forces can compensate for the cell-intrinsic forces normally produced by WAVE complex. This confined movement relies on a completely different mode of leading-edge advance, with processive bleb-based protrusions forming in a back-and-forth motion that extends the leading edge in a serpentine manner. Surprisingly, these serpentine protrusions coincide with Rac-based local positive feedback loops that set a permissive zone for bleb propagation, operate independently of branched actin assembly, and form a mechanistically distinct polarity circuit in this context. Finally, we find that the long-range inhibitor that underlies competition between protrusions does not always respond to membrane tension directly but may rely on closely linked properties such as global cell shape and local membrane curvature. Our work reveals a hidden circuit for neutrophil polarity and indicates that cells have distinct molecular mechanisms for polarization that dominate in different microenvironments.

P619/B634

The Ca^{2+}Channel ORAI1 Is Essential for Lamellipodia Formation and Directional Persistence in Osteosarcoma U2OS Cells.

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Cell migration requires coordinated regulation of the cortical cytoskeleton to promote the generation of plasma membrane protrusions at the leading edge that allows the formation of focal adhesions and cell progression. Epithelial tumor cells are dependent on the formation of persistent and polarized lamellipodia that allows directed cell migration. To generate lamellipodia, a protein complex known as the WAVE regulatory complex (WRC) promotes the assembly of actin filaments to push the front of the cell forward. This process is cyclic and the continuous formation and severing of actin filaments, known as plasma membrane ruffling, is dependent on Ca^{2+} influx. In order to determine the extracellular Ca^{2+}

entry route that allows this reorganization of the cortical cytoskeleton, we have studied the possible participation of the plasma membrane Ca^{2+} channel ORAI1 in this event. We have found that ORAI1 co-localizes and co-precipitates with WRC members in response to the activation of the small GTPase RAC1, an upstream activator of the WRC and downstream mediator of epidermal growth factor, EGF. By subcellular localization of ORAI1-GFP, we have found that RAC1 stimulates the translocation of ORAI1 to the leading edge increasing its externalization. This RAC1-dependent stimulation promoted the formation of lamellipodia, increased protrusions persistence and played a key role in cell directness, features that were sensitive to the pharmacological inhibition of RAC1 with NSC 23766. The genomic edition of U2OS cells to generate a U2OS ORAI1-KO cell line confirmed that ORAI1 mediates the influx of Ca^{2+} at the leading edge, as KO cells showed reduced lamellipodia, persistence, and directness in a random cell migration assay. Finally, we have demonstrated that ORAI1 has a role in the disassembly of F-actin and that this Ca^{2+} channel was essential for the binding of gelsolin to F-actin, a set of results that explain the reason for the need for ORAI1 at the leading edge.Funded by grants BFU2007-82716 (Spanish Ministerio de Ciencia, Innovacion y Universidad), IB16088 and GR18084 (Junta de Extremadura). All these grants were co-funded by European Regional Development Funds (ERDF/FEDER).

P620/B635

Store-operated Calcium Drives Calcium Sensor and Channel Trafficking to Regulate Filopodia.

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Filopodia are sensors for signals in the cell's microenvironment and set the direction for cell motility in several cell types. The experimental elevation of intracellular Ca^{2+} level could initiate either extension or retraction in the most widely used model system, the axonal growth cone. Here, calcium content and filopodia dynamics are investigated using conditions under which chemotactic and haptotactic gradients are obviated. Removal of serum caused an increase in filopodia, which was enhanced several-fold by simultaneous endoplasmic reticulum (ER) stress with cyclopiazonic acid. Whereas these increase in filopodia was transient, cytoplasmic calcium, $[Ca^{2+}]_i$, increased continuously during net filopodia retraction. Inhibitors of calpain or calmodulin canceled the return of filopodia to the starting point, so both of these proteins were negative effectors of Ca^{2+} signaling. Restoration of external Ca^{2+} caused a persistent increase in filopodia which was blocked by transient potential canonical (TRPC) channel inhibitors but not voltage-gated calcium or Ca^{2+} release-activated channel (CRAC) inhibitors. Immunofluorescence localizations of stromal-interacting molecule (STIM), Orai, and TRPC suggested they resided in different compartments in untreated cells. All three proteins were also in loci with different radii. Orai became localized with STIM at the cell surface during ER depletion. Clusters of TRPC1 were found on microtubules or free in the peripheral cytoplasm in untreated cells, but appeared as a continuous layer at the surface during ER refilling. The data suggested that the effects of high internal Ca^{2+} were opposite during ER depletion and refilling. The Ca^{2+} influx caused formation of TRPC-STIM complexes at the cell surface and promoted filopodia growth. The prevalence of filopodia was further increased by a Ca^{2+}/calmodulin inhibitor. The results suggest that calcium-dependent suppression of filopodia is overcome by Ca^{2+} influx and exocytosis of TRPC channels.

P621/B636

Local Ca^{2+} Signaling Via a Stretch-activated Channel Trpv4 Facilitates Lamellipodial Protrusions and Orchestrates Directional Cell Migration.

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Many biological processes, such as tissue morphogenesis and immune responses, require a coordinated, directional cell movement. To move directionally, cells require precise spatiotemporal regulation of force-generating components, e.g. Actin and myosin, focal adhesions, and regulatory proteins. Ca^{2+} is an important regulator of cell migration as indicated by the necessity of Ca^{2+} for cell polarization and protrusion-retraction cycles. Moreover, the dynamics of Ca^{2+} signaling have been shown to correlate spatiotemporally with lamellipodial protrusion events. Yet, it remains unknown which specific Ca^{2+} channel(s) control the structure and dynamics of cell lamellipodia and what molecular players are involved in this regulation.Using a genetically-encoded calcium biosensor, we showed that Ca^{2+} is enriched at the protruding edge of migrating fibroblasts. Inhibiting Ca^{2+} signaling was found to suppress lamellipodia extensions, perturb protrusion-retraction cycles of lamellipodia and impede cell migration. By combining siRNA gene silencing and cell spreading assay, we identified a mechano-gated Ca^{2+} channel TRPV4 as a prominent regulator of lamellipodia extension. We showed that TRPV4 is enriched in lamellipodia, suggesting that TRPV4 may regulate Ca^{2+} signaling at the cell edge. Inhibiting TRPV4 was found to decrease Ca^{2+} level at the protruding edge. To determine if TRPV4-mediated Ca^{2+} signaling regulates protrusion dynamics, we employed live-cell imaging and quantified the frequency and speed of protrusions. We found that suppression of TRPV4 resulted in slower and less frequent protrusions, suggesting that cell protrusion machinery is defective. Immunostaining of TRPV4-suppressed cells showed lower densities of actin network and nascent adhesion in lamellipodia.To understand the molecular link between TRPV4-dependent Ca^{2+} events and protrusions, we used an optogenetic inhibitor of Ca^{2+}/calmodulin-dependent protein kinase (CaMKII), a key Ca^{2+} signaling hub in many cell types. We found that local inhibition of CaMKII led to a significant reduction in the frequency and speed of protrusions, similar to those in TRPV4-suppressed/depleted cells. Inhibiting CaMKII showed a similar phenotype, suggesting that CaMKII is the mediator of Ca^{2+}-regulated cell protrusions. To determine the role of TRPV4-mediated Ca^{2+} signaling in directed cell migration, we assessed the speed and directionality of cell movement when either TRPV4 or CaMKII was suppressed and found that cell migration was impaired in both conditions.Together, these data unravel a new signaling axis composed of TRPV4/CaMKII, which regulates the structure and dynamics of protruding cell edge, enhances the efficiency of the protrusion machinery and orchestrates directed cell migration.

P622/B637

The Cadherin Fat2 Organizes a Planar Signaling System That Directs Actin Protrusion and Collective Migration of Epithelial Cells.

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Cells migrating as a collective need to ensure that their leading and trailing edges align with their neighbors, a form of planar cell polarity. We study how a novel planar cell polarity system in the Drosophila egg chamber epithelium coordinates one such collective migration. The follicle cells that make up the egg chamber epithelium undergo a highly coordinated collective migration along an underlying basement membrane extracellular matrix. The tissue lacks leader cells, and tissue-level polarity emerges from cell-cell interactions across the field. We show that Fat2, an atypical cadherin that

localizes to the trailing edge, promotes lamellipodial protrusion by recruiting the Wave complex to clusters at the leading edge of the cell behind. Fat2 also recruits Lar and Sema-5c, two transmembrane signaling proteins, to these leading edge clusters to form a planar-polarized intercellular signaling complex. Key next questions include 1) How does the Fat2 signaling complex organize asymmetrically across cell-cell junctions and 2) How does polarity information propagate across the cell field?

P623/B638

The Role of ERalpha36 and GPR30 receptors in the Estrogen Signaling in Inflammatory Breast Cancer.

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Inflammatory Breast Cancer (IBC) is a rare and very aggressive type of breast cancer; reporting a rapid progression and a poor prognosis compared to other subtypes of breast cancer. Approximately ~40% of IBC cases are classified as triple negative breast cancer (TNBC), due to the absence of hormone receptors and HER2 receptor; for which there is still no effective targeted therapy available. Estrogen (E2) have been found to enhance the migration and invasion phenotypes of IBC cell lines; by activating a novel cytoplasmic receptor (ERα36 isoform) and a membrane localized G-protein receptor (GPR30). However, this mechanism is not well characterized in IBC. Here, we evaluated the transcriptome changes in IBC cells upon exposure with E2 and/or GPR30 receptor inhibitor (G15). We hypothesized that ERalpha36 and GPR30, upon exposure with E2, are necessary to conduct transcriptome regulation in IBC cells; enhancing its oncogenic phenotypes. **Methods:** We performed Immunofluorescence microscopy and RNA-seq assay in these 4 study groups; ETOH-treated, E2-treated, G15-treated and E2-G15-treated IBC cell line SUM149PT (classified as TNBC), for ERα36 and GPR30 receptor detection and localization, and to identify specific transcriptome changes associated with oncogenic phenotypes. **Results:** Inhibition of receptor GPR30 was associated with biological processes such as: cell-cell adhesion via plasma-membrane adhesion molecules, regulation of membrane potential, and peptide hormone metabolism (adjusted p-value <0.01), among others. These results suggest that GPR30 is involved in the regulation of genes associated with the oncogenic phenotypes in IBC. Furthermore, by IF, we suggest ERα36 can translocate into the nucleus upon activation with E2. Data acquired from these experiments will contribute to the better understanding of IBC molecular signature and create new opportunities to develop novel targeted therapies. Xavier Bittman – xavier.bittman@upr.edu Josué Perez - joperez@cccupr.org Esther A. Peterson - esther.peterson@upr.edu Xavier S. Bittman Soto Xavier.Bittman@upr.edu 10-1-2019

P624/B639

Non-canonical Wnt Signaling Promotes Stem Cell Migration in the *Drosophila* Intestinal Epithelium.

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Adult stem cells play crucial roles in the maintenance and repair of high-turnover tissues. In multiple tissues, stem cells respond to damage and migrate from their current niche to the site of injury, proliferating or differentiating to replace lost cells. As such, stem cell migration must be carefully regulated to enable proper regeneration without disrupting the stem cell niche during homeostasis. In the *Drosophila* intestine, we find that intestinal stem cells (ISCs) are largely static under homeostatic, unchallenged conditions, but undergo lamellipodium formation and cell migration after intestinal damage by *Ecc15* bacterial infection or laser ablation. We find that this process is actin-dependent, and

regulated by non-canonical Wnt signaling. ISC migration is blocked after inhibiting Myosin II activity by blebbistatin treatment, or disrupting the actin-associated proteins, Dia and the Arp complex. Depletion of Otk (Ptk7 in mammals), a transmembrane protein in the non-canonical Wnt pathway, and its interactor, Dishevelled (Dsh/Dvl), prevents ISC migration after tissue damage, while overexpression of Otk and Dsh was sufficient to promote lamellipodium formation in unchallenged conditions. We also observe that depletion of Otk or Dsh decreases mitotic numbers, suggesting an additional role of these proteins in ISC proliferation. Altogether, we find that ISCs migrate in response to damage, and our data support a role for non-canonical Wnt signaling in regulating this behavior.

P625/B640

Negative Surface Charge Defines the State of Cell Cortex and Regulates Excitable Dynamics in Amoeboid Migration and Macropinocytosis.

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The spatially distributed components of signal transduction excitable network (STEN) and cytoskeletal excitable network (CEN) in Dictyostelium and leucocyte cells generate autonomous waves in the substrate attached basal surface. One key event in this characteristic cortical wave formation is the symmetry breaking of membrane phospholipids and self-organization of STEN and CEN proteins to dynamically generate distinct regions in the cell cortex which corresponds to the typical front (or “activated”) and back (or “inactivated”) regions of a randomly migrating cell. An analogous pattern of organization is observed in chemotactic migration, micropinocytosis, phagocytosis, and cytokinesis of different types of eukaryotes cells. We have identified two novel unexpected features of these self-organizing patterns. First, we have found that three lipidated membrane proteins in Dictyostelium, PKBR1, Gßγ, and RasG, which are widely thought to be uniformly distributed over the membrane, show preferential localization in the back/inactivated regions of cell cortex. Our data indicates that besides conventional recruitment based mechanism used by many other components, a dynamic partitioning process may contribute to the self-organization pattern of components that remain associated with the membrane. Second, we have assessed the localization of the generic surface charge sensor that was previously shown to be tightly associated with the negatively charged inner surface. In Dictyostelium and RAW 264.7 cells, we found that the sensor partitioned to the back/inactivated region as did the lipidated proteins. Our data suggested that high negative surface charge defines the back or inactivated region of the cell cortex and the cell migration and macropinocytosis processes are essentially guided by the alteration of surface potential on the inner leaflet to generate dynamic polarity.

P626/B641

Genetic Screening for Novel Partners of an Adhesion Regulator - Kinase Responsive to Stress B (KrsB).

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Dictyostelium discoideum social amoeba is a well-established model organism for the study of amoeboid-type migration, which is the type of movement seen in neutrophils and metastatic cancer cells. Cycling between active and inactive forms of the serine/threonine kinase responsive to stress B (KrsB), a homolog of mammalian tumor suppressor MST1/2 and Drosophila Hippo, contributes to the dynamic regulation of cell adhesion that is needed for proper cell adhesion and chemotaxis in D.

discoideum. However, the exact mechanism by which KrsB affects the cell's ability to adhere is unclear. The goal of this project is to find new regulators or effectors of KrsB using a genetic suppressor screen. Cells lacking KrsB were transformed with a cDNA library and mutants that exhibited either a rescue or an enhancement of the original phenotype were isolated. Cells lacking KrsB have a distinct phenotype when they form plaques on a bacterial lawn, with an enlarged region of cells in streams and an uneven expanding front of vegetative cells, which makes krsB$^-$ plaques appear to have rough edges. During the first round of screening, 150 plaques were identified, 48 of which showed a phenotype that was different from krsB$^-$: 30 plaques had smooth round edges similar to wild-type or cells rescued with KrsB, and the rest had phenotypes that differed from krsB$^-$ or wild-type cells, such as plaques that completely lacked aggregating cells. Thus, the first round of screening demonstrated that the cDNA library may provide genes that can compensate for the lack of KrsB. Efforts are underway to expand our collection of mutants and to isolate plasmids with the cDNA library inserts to identify the genes responsible for the rescue of the krsB$^-$ phenotype or for making the phenotype more severe. Identification of these genes will give us a better understanding of the molecular mechanism of KrsB function in cell adhesion and migration.

P627/B642

Integrating Neutrophil Fronts and Backs with the Mtorc2 Mechanotransduction Pathway.

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Neutrophils leverage feedback between mechanical forces and biochemical signaling to guide their polarity and motility during immune surveillance. For efficient movement, cells need to establish a single leading edge. We recently found that tension-based communication between actin protrusions (relayed by the PLD2/ mTORC2 pathway) are responsible for long-range competition between protrusions for proper control of polarity and movement. mTORC2 is an evolutionarily conserved regulator of cellular growth, proliferation and metabolism. Surprisingly, this signaling node has been co-opted for mechanochemical regulation of membrane homeostasis and cellular polarity or motility in systems ranging from yeast to Dictyostelium to neutrophils. Here we probe the molecular logic of how mTORC2 regulates the motility machinery. The kinase activity of mTORC2 is the most well-characterized route of regulating downstream effectors, but the complex is also thought to scaffold recruitment of some effectors in a kinase-independent fashion. Which of these activities links mTORC2 to the motility machinery is not well understood. Here we investigate this question using a combination of genetic and pharmacological approaches to selectively impair the kinase-dependent versus independent signaling roles of the complex. We find that the tension-based inhibition of Rac activation that enables competition between protrusions is gated by the kinase-independent role of the complex, whereas the mTORC2 kinase arm is essential for regulation of Myosin II activity at the trailing edge. With live cell imaging and Rac/Rho biosensors, we show the necessity of both of these branches of mTORC2 signaling for leading and trailing edge organization, cell polarity, movement, and guidance. Our results show how stretch activates the kinase dependent and independent arms of mTORC2 to integrate both the front (Rac) and back (Rho) polarity programs during neutrophil motility.

P628/B643

A Mechanistic Model of PLC/PKC Signaling Implicates Phosphatidic Acid as a Key Amplifier of Chemotactic Gradient Sensing.

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Chemotaxis of fibroblasts and other mesenchymal cells is critical for embryonic development and wound healing. Fibroblast chemotaxis directed by a gradient of platelet-derived growth factor (PDGF) requires signaling through the phospholipase C (PLC)/protein kinase C (PKC) pathway. Diacylglycerol (DAG), the lipid product of PLC that activates conventional PKCs, is focally enriched at the up-gradient leading edge of fibroblasts responding to a shallow gradient of PDGF (Asokan et al., PMID 25482883), suggesting that an internal amplification mechanism is at play. In previous work, a mechanistic, reaction-diffusion model of the PLC/PKC signaling pathway was developed to identify possible mechanisms responsible for signal amplification (Mohan et al., PMID 28700916). We found that phosphorylation of myrisotylated alanine-rich C kinase substrate (MARCKS) by membrane-localized PKC constitutes a positive feedback loop sufficient for local amplification of DAG and active PKC at the leading edge. However, by itself, the MARCKS feedback only weakly amplifies the signal in shallow PDGF gradients, and the system lacks robustness to modest changes in the midpoint concentration of PDGF. The new model includes phosphatidic acid (PA), a lipid intermediate in the metabolism of DAG, identifying roles in two other positive feedback loops. Model simulations show that the MARCKS feedback mechanism synergizes with these new feedback loops to polarize PKC signaling in response to shallow PDGF gradients and over an appreciable range of midpoint PDGF concentrations. DAG kinases and phospholipase D, the enzymes that produce PA, are implicated as key regulators, and testable predictions related to perturbation of DAG kinase activity are most promising.

P629/B644

Novel Extraction Method of Molecular Activity Patterns Controlling the Cell Morphological Changes Based on Reverse Correlation an alysis.

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Cell migration are one of the important biological phenomena related to various biological functions. The molecular mechanisms of cell migration have been extensively studied in tissue culture cells. Rho GTPases (Rac1, Cdc42, RhoA) have been identified as key regulators of cell migration(Alan Hall *Science*,1998). In previous work, we have measured and quantified the spatio-temporal signals of Rho GTPase activity and cell morphological change in migrating HT1080 cell by using the FRET imaging and quantitative imaging analysis. Furthermore, we have uncovered the relative timing between Rho GTPase activity and morphological change based on the time-shifted cross-correlation analysis (K Kunida et al., *J Cell Sci*, 2012). Cross-correlation analysis is powerful tool for investigating the relative timing between signals. But there are several concerns; (i)The dynamic relationship between signals cannot be seen in cross correlation. (ii)The obtained cross-correlation coefficient is very small (it is around 0.5 in our and other groups research). (iii) Positive or negative of the shift time at which the correlation coefficient is maximum does not necessarily indicate a causal relationship. From the above, this study proposes a new data analysis method for extracting dynamic relationship from sequential data of Rho GTPases activity and cell morphological change. In this study, we propose Motion-Triggered Average (MTA) as a method

to extract time-series data of molecular activity that control the specific pattern of morphological change based on reverse correlation analysis. Specific sequence patterns of resulting morphological changes are defined, and the average of molecular activity data having the same relative time to each morphological change is calculated to obtain molecular activity information from which noise is removed. As the result, we extracted the time-series of Rho GTPase activities corresponding to 4 typical cell migration patterns (wavelike-protrusion or retraction, directed-protrusion or retraction) with MTA analysis. Comparing with the random sampling, MTA analysis could extract signals with high similarity despite the different cell. Finally, we performed the Principle Components an alysis (PCA) for MTA-identified signals of Rho GTPase activities and confirmed that MTA analysis could identified distinct patterns of Rho GTPase activities.

P630/B645

Myosin IIA and Pi3k Are Important for the Basal Protrusion of Nrk52e Ras^{V12} Cells during Cell Competition.

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Cell competition describes a process by which a small number of unfit cells are eliminated by a vast majority of surrounding cells. In mammalian cells, the outcome of cell competition was mainly the apical extrusion of unfit cell from the cell layer. For the last decade, MDCK were the only mammalian cells used for the study of cell competition. Therefore, it is not known if this process occurs also in other mammalian cells. In this study, we show that cell competition can occur also in Normal Rat Kidney 52E epithelial cells (NRK52E). Interestingly, unlike the apical extrusion often observed in MDCK Ras^{V12} cells (unfit cells) surrounded by WT MDCK cells, NRK52E Ras^{V12}cells behaved in a totally different manner. We first demonstrate that when NRK52E Ras^{V12}cells are surrounded by normal cells, Ras^{V12}cells become highly motile and are fragmented. An d we show that this process is apoptosis-independent. We also demonstrate that this movement happens beneath the layer of normal cells. Previously it has been demonstrated that the elevation of myosin IIA in MDCK Ras^{V12}cells during cell competition is important for the apical extrusion of these cells. Interestingly, the treatment of NRK52E Ras^{V12}surrounded by WT cells by the myosin inhibitor Blebbistatin significantly increased the number of extruded Ras^{V12}cells. Moreover, the Knockdown of Myosin IIA in Ras^{V12}cells showed a similar result. Previous study has also demonstrated that Myosin IIA activation affects E-cadherin/catenin complex. We interestingly found that the expression of E-cadherin was depleted when Ras^{V12}is activated. To further investigate the mechanism of cell competition in NRK52E cells, we tried to inhibit different effectors downstream of Ras such as PI3K, p38, ERK, JNK and ROCK. Myosin IIA is usually activated by ROCK but its inhibitor Y27632 didn't block the basal protrusion of Ras^{V12}. Instead, we found that the treatment of NRK52E cells by the PI3K inhibitor LY294002 significantly increased the apical extrusion of Ras^{V12}cells surrounded by WT cells. Moreover, we show that the level of P-Akt is increased in Ras^{V12}cells. However, the LY294002 treatment doesn't seem to significantly decrease the level of P-Akt. This study shows that cell competition can also occur in other mammalian cells and that in this model Myosin IIA and PI3K are important for the basal protrusion of NRK52E cells independent of P-Akt.

P631/B646

Analysis of Rho Gtpase Networks Using Gtpase-trap Reveals That Rac-induced Contractility Changes Cell Migration Behavior.

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The Rho GTPases, Rac1, Cdc42 and RhoA, coordinate cytoskeletal dynamics and regulate cell motility through binding to various effectors. There have been many efforts to identify interactions between Rho GTPases and their effectors so as to understand the molecular mechanisms underlying cell migration. However, obtaining a highly refined, large-scale network of Rho GTPases has been challenging because most previous interaction assays were performed at a single GTPase level using experimental methodologies and conditions that varied. In this report, we developed a super-sensitive, large-scale interaction-screening method for use in live cells called 'GTPase-Trap'. Utilizing this method, we demonstrate that Rac proteins strongly bind to ROCK (Rho-associated kinase) proteins, which have long been known as typical downstream effectors of RhoA. Perturbation of Rac-ROCK interactions revealed that the contractility induced by this interaction is essential for lamellipodia protrusion, arc stress fiber formation, focal adhesion regulation, and cell migration behavior. Thus, the molecular network of Rho GTPases identified by our method provides new insight into the underlying mechanisms by which these GTPases coordinate actin cytoskeleton, focal adhesion dynamics, and cell migration.

P632/B647

Modeling Cell Repolarization in Single Cells and in Cell-cell Contacts.

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What external stimuli makes a polarized cell de-polarize or re-polarize? Under what conditions would a motile cell undergo contact inhibition of locomotion (CIL) when it encounters another cell? Would colliding cells adhere, stall, reverse or move together? to address these questions, we extend a basic model for cell polarization due to Mori et al (2008) Biophys J 94: 3684. The model describes the spatio-temporal distribution of GTPase activity by a "wave-pinning" mechanism. We first analyze polarity robustness and show its dependence on the total amount of GTPase and the rates of (in)activation by GAPs/GEFs. We show that some cell states are robust and refractory to repolarization, whereas other states are highly sensitive. We then add bi-directional feedback between cell forces and GTPase activity, and show that our simulated repolarization closely resembles experimental data (from opto-genetic manipulations of GTPases, and cell-cell collisions in microfluidic channels). Simulations in 1D kymographs, as well as 2D motile cells (with the Cellular Potts Model) are presented. We explore various assumptions for the link between cell-cell forces and GTPase signaling and how those assumptions affect paired cell migration. Our theoretical and simulation studies pave the way to investigate the roles of forces and signaling in collective migration of cell-trains and epithelial sheets.

P633/B648

Rap1 Regulates Adhesion and Cytoskeletal Rearrangements during Embryonic Wound Healing.

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Collective cell movements contribute to embryonic development and tissue repair, as well as the spread of disease. Cells coordinate their migration through adhesion and cytoskeletal rearrangements, but how

these molecular rearrangements are regulated is not well understood. We investigate collective cell migration during wound healing in the *Drosophila* embryonic epidermis, a process in which cells coordinate their movements to rapidly seal a lesion. After wounding, cells immediately surrounding the lesion internalize cell-cell adhesion molecules from the wound edge, including the core adherens junction (AJ) proteins E-cadherin, alpha-, and beta-catenin. AJ proteins are subsequently redeployed to the former tricellular junctions at the wound edge. In parallel, the cells polarize their actomyosin cytoskeleton to form a supracellular cable around the wound. Formation of the actomyosin cable requires the redistribution of AJ components at the wound edge. Through a combination of contraction of the actomyosin cable and reinforced adhesion, cells coordinate their movements to promote rapid wound repair. However, the signals that initiate and coordinate the molecular rearrangements that drive wound closure are unclear. The small GTPase Rap1 is a mechanosensitive molecular switch that promotes cytoskeletal polarization during collective cell migration in *vivo*. Rap1 also regulates cell junction turnover and interacts with actin regulators. Thus, Rap1 may coordinate and integrate the junctional and cytoskeletal rearrangements that drive wound repair. We tracked the dynamics of Rap1 and E-cadherin localization during wound closure, and we found that Rap1 was rapidly depleted from the wound edge and localized to tricellular junctions, preceding the redistribution of E-cadherin. These data suggest that Rap1 may be necessary for the molecular rearrangements that drive embryonic wound closure. Rap1 depletion by RNAi slowed down wound repair by 47% relative to controls, indicating that Rap1 is important for rapid embryonic wound healing. Additionally, polarization of the molecular motor non-muscle myosin II at the wound edge was 49% lower upon Rap1 RNAi treatment, suggesting that Rap1 mediates formation of the supracellular actomyosin cable around the wound. Similar results were obtained by overexpressing a dominant-negative form of Rap1. Together, our data suggest that Rap1 plays an integral role in regulating the adhesion and cytoskeletal rearrangements required for cells to coordinate their migration during embryonic wound healing.

P634/B649

Genetic Interaction between Adhesion Regulators Rap1 and Kinase Responsive to Stress B in *Dictyostelium Discoideum*.

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Cell adhesion to the substrate influences a variety of cell behaviors and its proper regulation is essential for migration. Social amoeba *Dictyostelium discoideum* is an extensively and commonly used model organism, whose movement is very similar to that of other amoeboid cells, such as neutrophils and metastatic cancer cells. Although we know many components of the signal transduction network that regulate directed migration, details of the pathways regulating cell adhesion during migration are lacking. Rap1 is a small GTPase that regulates adhesion in Dictyostelium cells in part via its effects on myosin II and talin. Kinase responsive to stress B (KrsB), a homolog of mammalian tumor suppressor MST1/2 and Drosophila Hippo, also regulates cell adhesion and migration, although the molecular mechanism of KrsB action is not understood. Since KrsB has been shown to interact with active Rap1 by mass spectroscopy, we decided to investigate the genetic interaction between Rap1 and KrsB. Cells lacking KrsB have increased contact with the substrate and are difficult to detach from the surface, which leads to reduced movement. Expression of constitutively active $Rap1^{G12V}$ increased cell adhesion, and inactive $Rap1^{S17N}$ reduced cell adhesion even in the absence of KrsB, suggesting that Rap1 does not require KrsB to mediate cell adhesion. However, $Rap1^{S17N}$ reduced cAMP-induced KrsB phosphorylation, whereas expression of $Rap1^{G12V}$ raised basal KrsB phosphorylation, suggesting Rap1 regulates KrsB

activation. Surprisingly, chemoattractant-induced activation of Rap1, as assessed by transient cortical localization of the biosensor RalGDS, was impaired in *krsB*$^-$ cells, possibly due to increased basal activity of Rap1. Thus, Rap1 appears to activate KrsB, which may function in a negative feedback loop to shut off Rap1 signaling, allowing for precise regulation of cell adhesion during migration.

P635/B650

The Novel Role of Phosphatidylinositol-3,4-bisphosphate in Cell Migration.

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Cell migration mediates a large number of key physiological activities during development and in the adult. These processes require coordination of signal transduction and cytoskeletal events which display excitability. Phosphoinositides have played a prominent role in signal transduction networks involved in migration, but the mechanisms of excitability are poorly understood. Here we identified that cells maintain complementary spatial and temporal distributions of Ras activity and Phosphatidylinositol-3,4-Bisphosphate (PI(3,4)P2) during random migration and in response to chemoattractants. Whereas Ras is active in the front, PI(3,4)P2 is distributed in a back to front gradient. In addition, depletion of PI(3,4)P2 by disruption of the 5-phosphatase, Dd5P4, or by recruitment of 4-phosphatase INPP4B to the plasma membrane, leads to elevated Ras activity, cell spreading and altered migratory behavior. Interestingly, lagging edge component PI(3,4)P2 localizes to leading edge protrusions, such as nascent macropinosomes, in a temporally and spatially restricted manner. PI(3,4)P2 enriched macropinocytic vesicles are transported into the cytosol and then break apart into “satellites” vesicles. Furthermore, using the green-to-red photoconvertible system, we determined that PI(3,4)P2 diffuses towards the front, suggesting there is a source at the back. Based on these observations, we propose there might be a connection between the PI(3,4)P2 on the front macropinosomes and back-to-front gradient of PI(3,4)P2 during cell migration.

P636/B651

Fine Tuning of the an ionic Membrane Lipids PI(4,5)P2 and Phosphatidylserine Establishes Polarized Morphologies and Regulates Cell Migration.

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Changes in cell morphology are critical for many cellular processes including cell division and the ability of cells to migrate. Many of the same signaling and cytoskeletal components regulate these morphological changes. Here we show that fine tuning of the plasma membrane (PM) PI(4,5)P2 levels and the rate of phosphatidylserine (PS) exposure contribute to a change in the charge of the inner leaflet of the PM and contributes to the feedback loops that set up polarized morphologies during migration. Synthetic lowering of PM PI(4,5)P2 levels below a threshold initiated positive feedback loops triggering actin polymerization and cell spreading. Conversely, raising PM PI(4,5)P2 levels significantly decreased cell speed and motility. Additionally, we demonstrate that PS is exposed on the outer leaflet of the PM in response to chemoattractants and after PI(4,5)P2 levels are lowered. This work shows for the first time a feedback loop between alterations in the PI(4,5)P2 pool on the inner leaflet and rates of PS exposure to the outer leaflet of the PM. Our work finds that there is a a gradient of PI(4,5)P2 in a polarized cell, with elevated levels at the rear. Taken together, these findings suggest that charge contributes to cell polarity, with migrating cells having a net loss of negative charge on the inner leaflet at the leading edge and an increase in negative charge at the rear of the cell.

P637/B652

Novel Role for Pikf in the Regulation of Pi(3,4)p_2 Dynamics.

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Directed cell migration is essential to a range of events including early organismal development and immune function. Migration is controlled by the highly coordinated activities of the cytoskeletal and signal transduction cellular networks. Phosphoinositides, a network of phosphorylated inositol lipids, serve as key regulators of the signal transduction networks involved in migration. Uncovering the networks of kinases and phosphatases which regulate phosphoinositide biosynthesis is vitally important to our understanding of the networks that regulate migration. Phosphatidylinositol (3,4)-bisphosphate [PI(3,4)P_2] has been found to antagonize movement by limiting Ras activity in a mutually inhibitory feedback loop. However, the identity of the molecules involved in mediating this mutual inhibition have not been fully described. The depletion of Dd5P4, a 5-phosphatase that generates PI(3,4)P_2, results in only a partial suppression of cellular PI(3,4)P_2 levels, suggesting the involvement of other unidentified enzymes in PI(3,4)P_2 biosynthesis. Here, we describe a novel role for pikF, a putative 3-kinase in *Dictyostelium,* that contributes to the balance of PI(3,4)P_2 biosynthesis. We demonstrate that pikF is a critical regulator of cellular protrusiveness and directed cell migration. We propose that a mutually inhibitory relationship between Ras and pikF dependent PI(3,4)P_2 biosynthesis serves as the mechanism of pikFs role in migration. Furthermore, we identify an important role for the formin-generated cortical actin network in the regulation of pikF dynamics. Our work provides important insight into the regulation of the cellular dynamics of PI(3,4)P_2, which is itself emerging as a critical regulator of cellular protrusiveness and directed cell migration.

P638/B653

CD56 (NCAM) Regulates Actin Cytoskeletal Function in Human Natural Killer Cells.

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Human natural killer (NK) cells are innate immune cells that are defined as being $CD56^+CD3^-$, however the functional role of CD56 (neural cell adhesion molecule, NCAM) on NK cells has not been described. We have previously shown that CD56 is required for human NK cell migration on developmentally supportive stromal cells. The objective of this study was to define the functional role of CD56 in regulating cell motility, adhesion and function in human NK cells. We deleted CD56 in two human NK cell lines using CRISPR-Cas9 gene editing and performed high- and super-resolution microscopy and scanning electron microscopy to define the effect of CD56 deficiency on the regulation of cell migration and the cytoskeleton. This was complemented by functional assays to measure the effect of CD56 deletion on lymphocyte effector function. We found that CD56 colocalizes with β1 integrin in the uropod of migrating NK cells, and that CD56 deficiency leads to impaired cell spreading. Cell migration in the absence of CD56 was inhibited due to impaired detachment of the cell uropod and actin architecture was highly deregulated. Structured illumination microscopy defined large actin asters at sites of focal adhesion proteins, suggesting that disassembly of integrin-mediated adhesion complexes is dysregulated. Impaired cell spreading was accompanied by increased filopodia length and disordered lamellipodia, underscoring the requirement for CD56 in actin polymerization and homeostasis. This was confirmed by scanning electron micrographs which revealed significantly longer filopodia and

deregulated membrane topology in resting cells in the absence of ligation; reconstitution of CD56 rescued these phenotypes. Finally, we tested immune synapse formation and NK cell lytic function. Despite the actin impairments in CD56-KO cells, they formed immune synapses with susceptible target cells but were unable to mediate target cell lysis due to impaired lytic granule exocytosis. This functional deficiency was accompanied by decreased phosphorylation and aberrant localization of the focal adhesion kinase (FAK) homologue Pyk2, a non-receptor protein tyrosine kinase that plays critical roles in lymphocyte migration and activation. Taken together, these data demonstrate a critical role for CD56 in human NK cell migration and function through modulation of Pyk2 signaling.

P639/B654

Pi-3 Kinase Drives Adaptive 3D Migration by Polarizing Large Pressure-based Protrusions.

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A hallmark of highly metastatic cells is the ability to navigate complex and diverse microenvironments. Cells with this capability often switch between motility modes, utilizing both actin-driven and pressure-driven protrusions to find or make empty spaces in dense extracellular matrices. In contrast to the belief that actin and pressure-based protrusion operate independently, we have discovered in this study that actin polymerization plays a critical role in reinforcing pressure-based protrusions to generate large blebs that push and remodel a 3D collagen network to create space for invading melanoma cells. We found that this localized protrusion reinforcement occurs through spatially restricted PI-3 Kinase (PI3K) signaling that drives actin polymerization through Rac1. By using a combination of high resolution light sheet microscopy along with 3D analytical tools, we show that regions of high PI3K create larger blebs. These blebs are reduced in size and redistributed in space under PI3K inhibition. By using photoactivatable forms of PI3K and Rac1, we found that bleb size can be increased by local activation of both PI3K and Rac1. Melanoma cells use these large pressure-based protrusions to dig tunnels through the soft but dense collagen network. Surprisingly, we found that this tunneling process does not require extracellular protease activity, but employs paxillin-containing adhesions along with repeated pressure-based “punches” to burrow through the network. Formation of adhesion structures at the front of these tunnels also localizes PI3K signaling to organize the large protrusions at the front of the cell where greatest contact occurs between adhesions and collagen. Classically, PI3K is thought to drive actin-based protrusion by recruiting Rac activators that generate a branched actin network. We also found actin inside growing blebs, suggesting that actin polymerization through Rac1 and PI3K drives bleb growth. This surprising result calls into question the very discrimination of actin-based and pressure-based protrusions and suggests that all protrusions may share common mechanisms. Furthermore, identification of PI3K as a common polarity cue explains how highly metastatic cells are able to switch protrusion mechanisms while maintaining directional polarity - cells use the same tools in different ways in order to switch protrusion mechanisms quickly when confronted with diverse microenvironments.

P640/B655

Let'S Stick Together: Defining the Molecular Mechanisms Underlying Chick Cranial Neural Crest Cell Epithelial to Mesenchymal Transition.

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Neural crest cells (NCC) are a transient, multipotent vertebrate cell population that differentiates into a variety of crucial cell types including melanocytes, chondrocytes, neurons or glia. Early in development, NCC undergo an epithelial to mesenchymal transition (EMT) in order to migrate and colonize different tissues throughout an embryo. EMT requires drastic changes to cell polarity, cell-cell adhesion, and the cytoskeleton. Currently, the molecular mechanism by which NCC undergo EMT is incompletely understood. Previous work in the lab identified a four-pass transmembrane protein, Tetraspanin18 (Tspan18), that antagonizes EMT in chick cranial NCC by maintaining the epithelial adherens junction protein, Cadherin 6B (Cad6B). In order for EMT to occur, Tspan18 and Cad6B must be downregulated. While Tspan18 and Cad6B mRNA expression is extinguished prior to EMT, the fate of Tspan18 protein in chick cranial NCC is unknown. Other cell types release tetraspanins in exosomes, a type of extracellular vesicle, and exosomes have known roles in the induction of EMT as well as promoting invasiveness and motility in cancer cells. It is unknown whether NCC release exosomes, and whether signaling through exosomes regulates NCC EMT. The aim of this project is to determine whether NCC release exosomes and the role of these exosomes in cranial NCC EMT. In order to identify NCC-derived exosomes, chick cranial NCC primary cultures were fixed and stained with the exosomal marker Hsc70 or live imaged using Bodipy Ceramide-TR (BC-TR). NCC expressed Hsc70 throughout the cytoplasm and in live imaging, movement of small, BC-TR labelled vesicular bodies suggests the presence of exosomes. Treatment of NCC cultures with 5 µM or 2.5 µM GW4869, a small molecular inhibitor of exosome biogenesis, resulted in differences in cell shape and migration distance between the treated and DMSO cultures. Together these data suggest that NCC release exosomes, and that exosomes are necessary for NCC migration. Future work will further characterize NCC-derived exosomes and their mode of signaling to define this previously unexplored method of NCC cell communication.

Intermediate Filaments

P641/B656

Identification of a Critical Phosphorylation Site That Regulates the Assembly of Glial Fibrillary Acidic Protein.

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Intermediate filament (IF) proteins form a major component of the cytoskeleton. There are >70 different human IF proteins, expressed in a cell type-specific pattern. GFAP is the major IF protein of mature astrocytes, important for providing structural support, regulating cell migration, and progression through mitosis. Naturally-occurring missense mutations compromise filament assembly and promote GFAP aggregation, impairing cellular function. Presently it is unclear how these mutations promote defective GFAP proteostasis. Our hypothesis is that naturally-occurring mutations interfere with GFAP filament assembly by disrupting critical post-translational modifications (PTMs). We used a computational approach to map known mutation sites on human GFAP in relationship to PTM sites that have been identified via proteomic studies, and curated by the PhosphoSite database. In parallel, we

performed mass spectrometry phospho-mapping analysis on GFAP extracted from post-mortem brain tissue of human subjects expressing either wild-type (WT) GFAP, or one of the following mutations: N77S, R79C, R239C, R239H, E373K, and D395Y. Based on the results of these analyses, we generated point mutants of GFAP designed to interfere with a key phosphorylation site, and analyzed their assembly properties in transfected cells via immunofluorescence analysis and in *vitro* via transmission electron microscopy (TEM). Our computational analysis of reported PTM sites on GFAP revealed that, in general, the modified residues fall outside of known mutation "hot spots." Specifically, we observed that phosphorylation sites were enriched in the N-terminal 'head' domain of GFAP (spanning residues 1-72), while the majority of GFAP mutations occur within the coiled-coil 'rod' domain (spanning residues 73-377) and C-terminal 'tail' domain (spanning residues 378-432). Furthermore, our mass spectrometry phospho-proteomic analysis revealed that phosphorylation of Serine-13 was highly enriched on human GFAP from subjects carrying missense mutations, but absent in GFAP from subjects expressing WT protein. This result was confirmed by western blot analysis using a phospho-specific antibody. The phospho-deficient mutant S13A formed abnormally short filaments compared to WT GFAP in *vitro* and in transfected cells, while the phospho-mimetic mutant S13D was completely incapable of filament assembly in *vitro,* forming uniform globular particles with an average size of 171nm^2. In transfected cells, S13D was significantly more sensitive to caspase cleavage and became incorporated into large perinuclear aggregates. In conclusion, our results reveal a shared PTM mechanism by which naturally-occurring mutations promote the destabilization of the GFAP intermediate filament network.

P642/B657

Hybrid Approach to Intermediate Filament Structure.

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The three-dimensional structure of 10nm wide intermediate filaments (IFs) remains an unresolved problem of structural molecular biology. Functional studies of various IF protein classes are flourishing due to their fundamental importance and scores of disease-related mutations found in IF proteins. Yet the understanding of their 3D structure is clearly lagging behind, aggravated by both intrinsic disorder and heterogeneity. In the past, we have achieved a substantial progress on the atomic structure of the signature central alpha-helical domain of IF proteins which forms a segmented coiled-coil dimer. Our recent crystallographic studies of keratins, vimentin and nuclear lamin a further highlight the dimer-dimer interactions responsible for the filament assembly process. In addition, we use chemical crosslinking coupled to mass-spectrometry analysis to elucidate the arrangement of the dimers in the assembly intermediates and in mature filaments, while cryo-electron microscopy is being exploited to unravel the overall structure of IFs assembled in vitro. Using such a panel of synergistic experimental approaches as well as 3D-modelling we gain substantial new insights into IF structure.

P643/B658

Vimentin Protects the Structural Integrity of the Nucleus and Suppresses Nuclear Damage Caused by Large Deformations.

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The mechanical properties of the cell and its nucleus are important factors in regulating cell migration through confined spaces. Early in migration, cells often initiate expression of vimentin intermediate filaments (VIFs), which extend from the nucleus to cell periphery. However, the role of VIFs in regulating nuclear shape and mechanics during cell motility is unknown. To examine this role, we used wild type (WT) and vimentin knock out (KO) mouse embryo fibroblasts (MEFs) and performed super resolution microscopy, Transwell migration assays, and atomic force microscopy (AFM). Our findings show that loss of VIFs affect nuclear shape and volume, and increases nuclear blebbing, rupture, and DNA damage during migration through constricting spaces. Image analyses revealed that VIFs are juxtaposed to the nuclear surface in WT cells, forming a cage-like meshwork surrounding it. Nuclei in WT MEFs were flatter and elliptical in shape compared to the KO cells that possessed rounder nuclei as determined by sphericity test. Average nuclear volume in KO MEFs was significantly smaller compared to the WT (475±13 vs. 631±23 μm^3, $p<0.0001$). Data from migration assays show that KO MEFs transit more readily through both pores of 3μm (5.5%±1.7% vs. 1.3%±0.6%, $p<0.01$) and 5μm (11.5%±3.8% vs. 2.55%±0.9%, $p<0.01$). The nucleus area was significantly smaller in WT and KO cells that migrated to the bottom of the membrane compared to those on the top (220±20 vs. 280±10 μm^2, $p<0.01$), yet WT and KO MEFs at the bottom had similar nuclear areas ($p>0.3$). Aspect ratio analysis showed that nuclear shape was altered in both cell types after migration ($p<0.01$), and that KO MEFs had significantly elongated nuclei compared to the WT ($p<0.001$). Translocation through the pores increased the fraction of nuclei with blebs in both cell types ($p<0.001$) with KO MEFs exhibiting 35% more nuclei with blebs than the WT ($p=0.03$). Migration through 3μm pores led to 50% increase in DNA damage repair foci in KO MEFs ($p<0.001$) compared to the WT. AFM results showed that WT MEFs were significantly stiffer in perinuclear region compared to the KO (0.46±0.035 vs. 0.33±0.04 kPa, $p<0.02$). Our results establish a new mechanism for the cytoskeletal resistance to nucleus deformation in cell migration in addition to previously demonstrated role for nuclear lamins. Supported by NIH GM P01 GM096971 and T32 CA080621-15.

P644/B659

Vimentin Intermediate Filaments Drive Cell Extension Formation and Cell Migration in a Substrate-dependent Manner.

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Cell migration involves adhesion to the extracellular matrix and actin filament reorganization, which result in the formation of specialized membrane extensions. Vimentin (VIM) intermediate filaments are centrally involved in cell migration during embryonic development, wound healing and cancer invasion. The direct interactions of actin filaments with the C-terminal tails of VIM, or indirectly through VIM binding partners, modulate cell adhesion receptors involved in cell migration. We considered that the impact of VIM on cell adhesion plays a crucial role in the formation and stabilization of cell extensions

and cell migration. VIM deletion in mouse embryonic fibroblasts (MEF) impaired cell spreading (two-fold; $p<0.01$), decreased the number and length of cell extensions (by 50% and 30%, respectively; $p<0.01$) and altered the localization of the extension tip marker, myosin 10 (MYO10). We found that VIM is required for MYO10 trafficking to the tip of cell extensions. Deletion of VIM promoted accumulation of MYO10 at the edge of the cell membrane (three-fold more compared to WT cell; $p<0.001$). As VIM evidently affects cell extension formation in a substrate-dependent manner, we quantified the impact of VIM expression on cell adhesion to collagen or fibronectin. VIM deletion increased (by 60%; $p<0.01$) fibronectin-coated bead binding compared with collagen binding. Deletion of VIM increased the abundance of total and activated β1 integrin receptors in cells plated on fibronectin. Finally, a phospho-site screen (VIM S39, S56, and S72) indicated that phosphorylation and spatial reorganization of the vimentin is higher on fibronectin (by 60%; $p<0.01$) than collagen what may determine differences in cell motility. In conclusion, our results demonstrate a central role of VIM in the regulation of cell extension formation and stabilization, which affects cell migration.

P645/B660

Keratin 19 Role in Mcf7 Breast Cancer Cells Morphology and Migration.

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Tumor metastasis is associated with epithelial to mesenchymal transition whereby an epithelial cell loses its polarity, cell to cell attachment, and altered expression of keratin intermediate filament proteins. Keratins regulate the shape and mechanical integrity of epithelial cells. Among keratins, keratin 19 is frequently overexpressed in breast cancer and is associated with poor prognosis. We found that K19 plays a role in maintaining epithelial cell shape and tight cell to cell adhesion in MCF7 breast cancer cells, as K19 depletion leads to elongated cell shape and loose cell to cell adhesion. Moreover, K19 depletion increases the migratory potential of MCF7. At the molecular level, E-cadherin, an actin associated adhesive molecule, was surprisingly found to be overexpressed in K19 depleted cells. However, we found that E-cadherin is less membrane associated and less stable in K19 depleted cells. Our results suggest that K19 promotes destabilization of E-cadherin, decreased cell adhesion and increased cell migration.

P646/B661

Vimentin Intermediate Filaments and F-actin Form Interpenetrating Networks in the Cell Cortex.

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Vimentin intermediate filaments (VIFs) and F-actin are both filamentous cytoskeletal proteins in the cytoplasm but they are typically understood to have vastly different properties and functions. However, there is some evidence that VIFs are involved in cellular functions commonly attributed to actin, which necessitates a closer look at the interplay between the F-actin and VIF cytoskeletons. Using structured illumination microscopy, we image the cortical region of mouse embryonic fibroblasts (MEFs), where we observe close associations between VIFs and F-actin bundles (stress fibers). Furthermore, cryo-electron tomography (cryo-ET) of stress fiber ultrastructure reveals that VIFs are pervasive throughout the structure, suggesting that a stress fiber is an interpenetrating network (IPN) of F-actin and VIFs.

Although many F-actin structures are facilitated by crosslinking proteins, we show that a mixture of purified actin and vimentin proteins self-assembles in vitro into an IPN without the help of binding proteins. Finally, we study the functional consequences of having composite VIF-actin structures in cells, and find that VIFs can significantly impact actin dynamics. We use traction force microscopy (TFM) to find that VIFs play an important role in cell contractility even though they are not intrinsically a force-generating unit; this contribution is likely due to the integrated structure. In addition, fluorescence recovery after photobleaching (FRAP) measurements reveal that VIFs hinder cytoplasmic G-actin diffusion. From these observations, we infer parallels with double-network hydrogels, which are tougher than either of their component gels. These results indicate that the interplay between VIFs and F-actin is an important aspect of cell mechanics, especially as it relates to the contractile nature of cells. These results demonstrate that VIFs play a broad and essential role in cellular mechanics by influencing both the structure and dynamics of actin. Supported by a NIGMS grant (P01 GM096971) awarded to R.G. An d D.W. An d a Swiss National Science Foundation grant awarded to O.M.

P647/B662

Expression of the N-terminal TRSEEX Domain of Bag-1M Prevents Binding to Intermediate Filaments.

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Bag1 (Large, Medium, or Small) isoforms are translated from a single mRNA with multiple start codons. Bag-1L and M share TRSEEX repeats in the N-terminal region, which are not present in Bag-1S. Bag1-L also displays a nuclear localization signal (NLS), responsible for nuclear import, not expressed in Bag1-M. Accordingly, the latter is strictly cytoplasmic. In previous work we demonstrated that Bag-1M binds to intermediate filaments (IF), and blocks IF-bound Hsp70 which participates in PKC refolding. Furthermore, Bag1 upregulation induced by TNF-alpha and IFN-gamma resulted in decreased levels of atypical PKC. We hypothesized that TRSEEX repeats must be involved in binding of Bag1-M to IF. We used two synthetic peptides with only one TRSEEX repeat, or the full Bag1-M N-terminal TRSEEX domain, but without the NLS. Both peptides included a C-terminal antennapedia cell-penetrating peptide, known to be effective in Caco-2 intestinal epithelial cells. In the same cell line, we also expressed the Bag1-M TRSEEX domain or the full Bag1-M ORF using stable lentiviral transduction. Both synthetic peptides and the lentiviral expression of the TRSEEX domain reduced nearly 80% the amount of Bag1-M attached to IF. Interacting partners of the TRSEEX domain were determined by BirA-mediated near neighbors biotinylation. Interactions of the Bag1-M N-terminal domain were observed on IF and in neighboring apical membrane compartments. As expected, expression of the TRSEEX Bag1 domain abrogated the post-translational decrease in aPKC induced by proinflammatory signaling. We conclude that this Bag1 N-terminal domain indeed mediates Bag1-M localization to IF but it is also localizes to intracellular membranes.

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Exploring the Intermediate Filament Proteome.

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Intermediate filaments (IFs) are perhaps the most complex, but least well studied, part of the metazoan cytoskeleton. IFs are known to play important roles in cell structure and mechanical support, adhesion

and migration, polarization, and organelle positioning, but also in cell signaling and cytoskeletal crosstalk. IFs are assembled from a diverse family of proteins, the majority of which are expressed in a cell type specific and/or developmentally regulated manner. Therefore, IFs tend to vary greatly in composition, as well as structure and function, ultimately providing each cell type with a unique cytoskeletal signature. Further diversification is achieved by posttranslational modifications and interactions with IF associated proteins (IFAPs). Mutations in genes encoding IF proteins are involved in a number of human diseases, with highly variable pathology. The Cell Atlas, which is part of the Human Protein Atlas (HPA) open access database, aims at providing high-resolution insights into the spatio-temporal distribution of proteins in human cells. Using antibody-based profiling by immunofluorescence and confocal microscopy, we have identified 191 candidate proteins that localize to IFs, including 150 proteins for which there is no previous experimental evidence for localization to this subcellular compartment in the UniProt database. Some of these are known members of the IF protein family, but the majority represent new potential IFAPs, including 18 uncharacterized proteins. Interestingly, as many as 72 of the proteins that localize to IFs display cell-to-cell variation in protein levels, when assayed by staining intensity. This variation may further reflect the complexity of intermediate filament structure and function, ultimately reflecting highly dynamic and responsive cell populations. In this study, we are presenting an overview of the intermediate filament proteome and aim at further characterizing the observed single cell variability among these proteins. This analysis of the IF proteome can provide a valuable knowledge resource for studies of IF function in human health and disease.

P649/B664

The Role of Septins in Platelet Morphology and Functions.

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Platelets are anucleate blood cells that play a role in hemostasis and thrombosis. Activated platelets change shape due to reorganization of cytoskeleton, consisting mainly of actin, tubulin, spectrin, and filamin. Septins, a family of GTP-binding cytoskeletal proteins, are implicated in division of nucleated cells, but their role in platelets remains unknown. Here, we used a combination of high resolution confocal microscopy, biomechanical and biochemical assays to study the distribution of septins 2 and 9 in resting and activated platelets and to explore how disruption of septin assembly impacts platelet morphology and physiology, including contractility, aggregation, adhesion, and integrity. As detected by immunostaining, in resting platelets septin 2 was concentrated almost exclusively around the cell periphery, forming a ring-like structure, while septin 9 formed small patches throughout the cell volume, often with peripheral localization. In platelets activated by thrombin, septins were also present in filopodia. Incubation of platelets with thrombin for longer times (1 hour) caused significant changes in septin organization, leading to a 2-fold increase of septin staining intensity, which was concurrent with a decrease of platelet size due to fragmentation. Impairment of septin dynamics with forchlorfenuron (FCF) caused dose-dependent alterations in platelet shape and functions. First, incubation of platelets with FCF for 1 hour resulted in a substantial decrease of platelet roundness and solidity parameters that quantify cell shape changes. Second, flow cytometry-based analysis of resting platelets treated with FCF indicated a significant 7- to 10-fold increase of the fraction of platelets expressing phosphatidylserine,

activated integrin αIIbβ3, and P-selectin. In FCF-pretreated platelets activated with thrombin-receptor activating hexapeptide (TRAP-6), expression of both phosphatidylserine and activated αIIbβ3 was significantly suppressed, while P-selectin was overexpressed compared to activated cells untreated with FCF. Third, treatment of platelets with FCF impeded platelet-driven clot contraction kinetics with a 6-fold increase of the lag-time and up to a 3-fold decrease of the extent of contraction. At later stages of platelet activation with thrombin, disruption of septin assembly with FCF resulted in a significant acceleration of platelet fragmentation. Meanwhile, FCF did not alter platelet aggregation kinetics induced by TRAP measured with light transmission aggregometery. Our findings suggest that septins play an important role in platelets by stabilizing their shape and maintaining platelet integrity; septins are also involved in the transmission of contractile forces during blood clot contraction.

Tight Junctions

P650/B666

Calcium Signaling Contributes to Epithelial Tight Junction Remodeling during Cell Shape Changes.

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Cell shape changes challenge epithelial cell-cell adhesion and barrier function. In order for an epithelium to serve as an effective barrier, tight junctions must be remodeled during events that cause cell shape changes, such as morphogenetic movements and cytokinesis. Using live imaging in the epithelium of gastrula-stage *Xenopus laevis* embryos, we have shown that junction elongation correlates with local loss of tight junction proteins, resulting in a leaky barrier. These leaks are rapidly repaired by localized, transient activation of RhoA - termed “Rho flares” (Stephenson et al., 2019) - which promote actomyosin-mediated reinforcement of tight junction proteins. However, the mechanism underlying local activation of Rho flares is unknown. Here, we show that a transient increase in intracellular calcium (Ca^{2+}) precedes activation of Rho flares and is localized to the site of Rho flares. Further, our data shows that intracellular Ca^{2+} increase is required for activation of Rho flares and efficient reinforcement of tight junction proteins. Because Rho flares are associated with junction elongation and apical plasma membrane deformation (Stephenson et al., 2019), we propose that a mechanosensitive channel, Piezo1, may be responsible for Ca^{2+} influx at the site of Rho flares. However, the localization of Piezo1 in the *Xenopus* epithelium and its potential function in regulating Ca^{2+} influx during tight junction repair are unknown. We find that Piezo1 is expressed in the gastrula stage *Xenopus* epithelium and localizes to apical cell-cell junctions. Both a broad mechanosensitive channel inhibitor (Gadolinium chloride) and a more specific Piezo1 inhibitor (GsMTx4) attenuate externally-induced Rho flare activation and Ca^{2+} influx. Interestingly, partial knockdown of Piezo1 increases the frequency of barrier leaks, alters Ca^{2+} influx dynamics, and causes abnormally large Rho flares. Together, our results suggest that mechanically-triggered Ca^{2+} influx through Piezo1 mechanosensitive channels may be an important feature of the mechanism by which cells sense and repair transient leaks in barrier function. Reference:

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P651/B667

Claudin-specific Interactions at the Tight Junction Cytoplasmic Plaque.

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Tight junctions are essential to regulate paracellular transport as well as to coordinate morphogenesis during development. Our lab showed that claudins, the main components of tight junctions, are essential for the proper elevation and fusion of the neural folds during neural tube closure. In fact, the removal of Cldn3, -4 and -8 through treatment with the C-terminal domain of *Clostridium perfringens* enterotoxin (C-CPE) generated open neural tubes and convergent extension defects in 100% of the embryos treated. Claudins are composed of a cytoplasmic tail, two extracellular and four transmembrane domains. They are known to interact with other claudins in the same or apposing cell using the extracellular or transmembrane domains. On the other hand, the cytoplasmic tail has been proposed to interact with different scaffolding proteins and cytoskeleton. However, a direct experiment has not been performed to identify specific interactors for each claudin. In our study, we used the cytoplasmic C-terminal domains from different claudins to identify claudin-specific interaction partners for Cldn1, -3, -4, -8 and -14 from different stages of embryogenesis (mid-neurulation, late neurulation and early organogenesis). We observed different interaction profiles for the individual claudins at the different embryonic stages. We hypothesize that this reflects the claudin-specific functions and dynamic expression patterns during development.

P652/B668

Lights, Camera, Rho Flare Action: Inducing Contractility with Optogenetics.

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Epithelial tissues generate barriers that compartmentalize multicellular organisms, support homeostatic nutrient and water transport, and prevent pathogens from invading the tissue. Epithelial cell-cell junctions, including tight junctions (TJs), which seal the paracellular space between cells, and adherens junctions, which promote cell-cell adhesion, are connected to an apical bundle of F-actin and Myosin II (actomyosin). Cell shape changes driven by dynamic events including cell division, wound healing, and developmental morphogenesis generate tension along junctions, which challenges cell adhesion and barrier function. We have shown that “Rho flares”, short-lived, local accumulations of active Rho, rapidly repair local leaks in barrier function *via* local actomyosin-dependent contraction of the junction[1]. However, the mechanism by which Rho flares are triggered remains unknown. Because Rho flares are preceded by junction elongation and a local loss of TJ proteins, we hypothesize that junction elongation may act as a mechanical trigger that initiates TJ leaks and the Rho flare repair mechanism. In order to test this hypothesis, we have adapted the previously-described TULIP optogenetic system, which locally activates contractility upon light stimulation[2], for use in live *Xenopus laevis* embryos. We have shown that whole-field light stimulation elicits recruitment of a photo-recruitable GEF followed by RhoA activation at cell-cell junctions in *Xenopus* embryos. We also observe cell shape changes (e.g. junction straightening) associated with actomyosin contractility upon whole-field stimulation. We aim to use targeted light stimulation of TULIPs to elongate junctions on demand with high spatiotemporal precision. This system will allow us to test whether junction elongation triggers local TJ leaks and Rho flares and will provide a platform for us to further investigate the mechanism of Rho flare activation. (1) Stephenson, Rachel E., Tomohito Higashi, Ivan S. Erofeev, Torey R. Arnold, Marcin Leda, an drew B.

Goryachev, and an n L. Miller. "Rho flares repair local tight junction leaks." *Developmental Cell* 48:4 (2019): 445-459. (2) Strickland, Devin, Yuan Lin, Elizabeth Wagner, C. Matthew Hope, Josiah Zayner, Chloe an toniou, Tobin R. Sosnick, Eric L. Weiss, and Michael Glotzer. "TULIPs: tunable, light-controlled interacting protein tags for cell biology." *Nature Methods* 9:4 (2012): 379.

P653/B669

Bicellular and Tricellular Tight Junction Molecules Localize to Midbody and Centrosome during Cytokinesis in Human Endometrial Carcinoma Cell Line.

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Epithelial integrity and barrier function are maintained during cytokinesis in vertebrate epithelial tissue. The changes in localization and the roles of the tricellular tight junction molecule lipolysis-stimulated lipoprotein receptor (LSR) during cytokinesis are not well known, although new tricellular tight junctions form at the flank of the midbody during cytokinesis. In this study, we investigated the changes in localization and the role of LSR at the midbody and centrosome during cytokinesis using human endometrial carcinoma cell line Sawano, comparing the tricellular tight junction molecule tricellulin, bicellular tight junction molecules occludin, claudin-7, ZO-1 and cingulin and the epithelial polarized related molecules ASPP2, PAR3 and YAP. During cytokinesis induced by treatment with taxol, the epithelial barrier was maintained and the tricellular tight junction molecules LSR and tricellulin were concentrated at the flank of the acetylated tubulin-positive midbody and in γ-tubulin-positive centrosomes with the dynein adaptor Hook2, while the other molecules were also localized there as well. All the molecules disappeared by knockdown using siRNAs. Furthermore, by the knockdown of Hook2, the epithelial barrier was maintained and most of the molecules disappeared from the centrosome. These finding suggest that bicellular and tricellular tight junction molecules may play crucial roles not only for barrier function but also for cytokinesis.

P654/B670

ZO-1 in Epithelial Morphogenesis:a Regulator and Transducer of Mechanical Force.

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Epithelia are cell sheets that separate body compartments by forming barriers. Epithelial morphogenesis is a process that drives development and puts tissues under physical constraints. Adaptations in cell-cell and cell-extracellular matrix (ECM) tension are necessary to maintain barrier function during this process, but the mechanisms balancing these forces are not well understood. The tight junction protein ZO-1 plays a central scaffolding role and tethers the actin cytoskeleton to transmembrane cell-cell adhesion proteins. We hypothesized that ZO-1 plays a central role in the balancing of mechanical forces by transducing cell-cell tension from the neighbouring cells to the cytoskeleton and by spatially regulating actomyosin activity through biochemical signalling. To validate our hypothesis, we used RNAi against ZO-1 in MDCK cells grown on different matrix stiffness to model varying monolayer tension conditions. Using a ZO-1-based FRET tension sensor, we observed that tension at tight junctions in MDCK cells correlated with ECM stiffness. Forces acting on E-cadherin were regulated by ZO-1 in a substrate stiffness-dependant manner. On stiff matrix, ZO-1 knockdown induced a flattened cell morphology with partially disrupted cell-cell junctions and increased tension on E-cadherin. The cell cytoskeleton was also affected: RhoA-GEFs, actomyosin and focal adhesions redistributed, which

correlated with increased F-actin polymerisation and myosin II phosphorylation. On soft substrates, tension decreased in ZO-1-depleted cells and traction force microscopy revealed reduced substrate traction upon ZO-1 depletion, which confirmed the decrease in cytoskeletal tension. On even softer substrates, ZO-1 depletion only led to a reduction in the cells capability to form continuous monolayers and disrupted lumen formation in 3D culture. We then used RNAi to identify ZO-1 transmembrane binding partners that could transduce tension and observed that JAM-A knock-down led unexpectedly to increased tension at tight junctions with no significant impact on adherens junctions. Finally, depletion of ZO-1 also impacted on transcriptional pathways regulated by tight junctions, leading to activation of YAP/TAZ signalling via a biochemical mechanism rather than tension-induced activation. To summarise, we demonstrate that crosstalk between apical and basal adhesion dynamics in epithelia is regulated by ZO-1 in a matrix-stiffness-dependant manner by mechanisms that involve biochemical signalling and transduction of tensile force across ZO-1.

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Activation of the Ca^{2+} Sensing Receptor and the PKC/WNK4 Downstream Signaling Cascade Induces Incorporation of ZO-2 to Tight Junctions and Its Separation from 14-3-3.

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Tight junction (TJ) protein ZO-2 displays a diffuse cytoplasmic distribution in cells cultured in low calcium (LC, 1-5 µM). However, if in confluent MDCK cells cultured in LC, the Ca^{2+} sensing receptor (CaSR) is activated with Gd^{3+}, ZO-2 appears at the cell borders. Here we have explored the signaling cascade triggered by the activation of the CaSR that leads to ZO-2 movement from the cytoplasm to the cell borders. CaSR is a G coupled receptor whose signaling through $G\alpha_{q/11}$ leads to activation of PKC. Accordingly, in cells cultured in LC, ZO-2 appears at the cell borders after treatment with DiC8, a permeable analogue of diacylglycerol that stimulates novel and conventional PKCs. Next we observed that treatment of LC cultured cells with bryostatin an stimulator of nPKC δ and ε, or with Gd^{3+} and röttlerin, an inhibitor of nPKC δ, allows the arrival of ZO-2 to the cell borders, thus indicating that activation of nPKCε is sufficient to induce ZO-2 relocation to the cell borders. Since PKC phosphorylates and activates with no lysine kinase-4 (WNK4), which favors TJ formation, we next analyzed the impact of bryostatin or DiC8 treatment on ZO-2 in LC cultured cells, observing with a proximity ligation assay (PLA) and immunoprecipitation assays that ZO-2 phosphorylation at serine residues augmented as well as ZO-2/WNK4 interaction, while inhibition of WNK4 with WNK463 blocked cell appearance at the cell borders induced by Gd^{3+} or DiC8, as well as ZO-2 phosphorylation at serine residues after DiC8 treatment. In addition, we observed that in LC, ZO-2 is protected from degradation by association to 14-3-3 proteins, and that stimulation of CaSR with Gd^{3+} diminishes the cytoplasmic co-localization of the ZO-2/14-3-3 complex. U2 region of ZO-2, and S261 located within a nuclear localization signal, are critical for the interaction with 14-3-3 ζ and σ and for the efficient nuclear importation of ZO-2. In summary, our results reveal the appearance of ZO-2 at the membrane triggered by activation of the CaSR/PKCε/WNK4 signaling pathway and the subsequent the disassembly of the cytoplasmic ZO-2/14-3-3 complex.

P656/B672

Rho Flares Rapidly Repair Local Breaches in Tight Junctions.

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An important function of epithelial tissues is to create biological barriers, thereby generating an internal environment distinct from the external environment of an organ or organism. Tight junctions play a key role in regulating the strength and selectivity of this barrier. However, many epithelial tissues are routinely subjected to intrinsically and extrinsically generated mechanical forces, such as those resulting from morphogenesis, cell division and extrusion, or normal organ function (e.g., bladder filling). To determine the degree to which these events challenge barrier integrity, we combined high spatiotemporal imaging in the *Xenopus laevis* embryo with ZnUMBA (Zinc-based Ultrasensitive Microscopic Barrier Assay), an assay that allows us to monitor subcellular changes in barrier function in real time. Using this approach, we found that local, transient breaches of tight junctions are caused by events that elongate junctions, such as cell division. These localized leaks are followed by Rho flares, sites of locally activated Rho GTPase, which repair the leak and locally increase tight junction and adherens junction proteins, such as ZO-1, occludin, and E-cadherin. We find that actomyosin-mediated junction contraction is critical for efficient leak repair and reinforcement of the tight junction. Rapid repair of the tight junction following cell shape change allows epithelial tissues to tolerate changes to cell shape with minimal impact on barrier integrity. Ongoing work focuses on the role of specific components of adherens junctions, tight junctions, and the cytoskeleton in facilitating efficient barrier restoration.

P657/B673

Nanoparticles That Interact with Beta 1 Integrin Promote Junctional Rearrangement at a Distance Due to Cytoskeletal Rearrangement Depending on Their Geometry.

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Epithelia form selective barriers that compartmentalize organs which allows for specialized physiologic function. Epithelial barrier permeability has two regulated components, the transcellular path mediated by transcytosis, and the paracellular path regulated by tight junctions (TJs). We previously determined that contact between the apical plasma membrane and nanostructured films (NSFs) increases the permeability of epithelial cells to large macromolecules, such as Alexa-594 Fab, through both the transcellular and paracellular pathways. Several lines of evidence identified a role for apically localized beta 1 integrin in the ability of NSFs to increase substrate permeability. However, a drawback to using NSFs is that they act by engaging large, heterogeneous patches of the apical plasma membrane making it difficult to precisely define molecular mechanisms linking specific surface proteins to changes in epithelial barrier function. Thus, to specifically investigate whether beta 1 integrin is directly involved in increased barrier permeability, we developed a nanowire system consisting of anti-beta 1 integrin antibodies conjugated to functionalized polycaprolactone nanowires. These anti-integrin nanowires serve as a platform to specifically cluster apically localized beta 1 integrin to determine whether integrin stimulation has the capacity to regulate epithelial barrier function. Treatment of epithelial monolayers with anti-integrin nanowires significantly decreased the transepithelial resistance of the monolayer and increased the rate of transepithelial flux of Alexa-594 Fab across monolayers. These functional effects were associated with nanowire-induced changes in the localization of the TJ scaffolding protein zonula

occludens-1 (ZO-1) as well as rearrangement of the actin cytoskeleton from stress fibers into a more cortical pattern of organization. Recently we have observed that the majority of these changes in ZO-1 localization do not occur in regions where the nanowires are in direct contact with ZO-1, but rather the anti-integrin nanowires induced these changes at a distance. Additionally, we have observed that the shape and size of the antibody conjugated nanoparticles plays a role in the severity of the barrier function response. Longer nanowires (15um x 200nm) have a more pronounced effect on barrier permeability, while shorter nanowires (5um x 200nm) have a more pronounced effect on ZO-1 localization. Anti-integrin microspheres (2um) have a moderate effect on both permeability and TJ structure. These data show that clustering apical beta 1 integrin does not have a local effect on TJ barrier structure, and that the regulation of transepithelial permeability via anti-integrin nanoparticles is dependent on nanoparticle geometry.

P658/B674

Probing the Molecular Architecture of Claudin-based Tight Junction Strands.

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Models of molecular architecture of tight junction (TJ) strands and paracellular channels have been proposed. However, they were not fully consistent with experimental and structural data. Here, we analyzed architecture of claudin-based TJ-strands and channels by cellular reconstitution of strands, structure-guided mutagenesis, in *silico* protein docking and oligomer modeling. Prototypic channel- (Cldn10b) and barrier-forming (Cldn3) claudins were analyzed. FRET-assays indicated multistep claudin polymerization, starting with claudin subtype-specific *cis*-oligomerization and extending by *trans*-interaction-mediated *cis*-polymerization. Alternative protomer interfaces were modeled in *silico* and tested by cysteine-mediated crosslinking, confocal- and freeze fracture electron microscopy-based analysis of strand formation. The results indicated antiparallel double row arrangement of protomers in Cldn10b- and Cldn3 strands, similar as suggested previously for Cldn15. Hydrophilic and hydrophobic *cis- and trans*-interfaces containing extracellular segment (ECS) 1 and ECS2 residues were defined. Cldn10b and -3 are indicated to share this polymerization mechanism. However, electrostatic repulsion (Cldn10b) or attraction (Cldn3) in the paracellular center of tetramers leads to pore- or barrier formation, respectively. Together, the study provides improvement of the molecular and mechanistic understanding of paracellular permeability regulation and its pathogenic alteration.

P659/B675

The Role of ATPIF1 Protein for Skin Barrier Function.

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The skin barrier is essential to maintain homeostasis and prevents water loss from the skin inside. The tight junction transmembrane proteins are known as important regulators on skin barrier function. A number of tight junction proteins including claudins, occludins and junctional adhesion molecules (JAMs) have been shown to form a barrier in the epidermal keratinocytes, the predominant cell type in the skin epidermis. In the present study, we identified new role of ATPase inhibitory factor 1 (IF1), an F1F0-ATP synthase inhibitor protein in improving skin barrier function. IF1 is a physiological inhibitor for

mitochondrial F1F0-ATP synthase with its binding activity. F1F0-ATP synthase is mainly located within the mitochondrial matrix; however, its F1 catalytic subunit β-F1-ATPase may also be localized on the plasma membrane of keratinocytes. So it is possible that cell surface F1-ATPase, namely ecto-F1-ATPase is involved in a different biological function and exogenous IF1 administration can act as a ligand for F1-ATPase to induce specific cellular responses. We observed that human epidermal keratinocytes (HEKs) exposed to recombinant IF1 protein underwent concentration-dependent cell proliferation as analyzed by WST-1 cell proliferation assay. The expression of tight junction proteins such as claudin-1 and JAM-A was also increased by IF1 protein treatment in a concentration-dependent manner. IF1 protein treatment significantly increased the transepithelial electrical resistance (TEER) of keratinocytes, indicating intact barrier function as electrical resistance across a cellular monolayer and prevention of transepidermal water loss. To confirm the cellular integrity, paracellular permeability was measured with in vitro transwell system using 4 kDa FITC-dextran was measured in keratinocytes. IF1 protein treatment decreased keratinocyte cell permeability 60% compared with the control group. Our findings provide new insight into the role of IF1 of the skin barrier formation, and may contribute to a potential therapeutic target for therapy of epidermal barrier defects.

P660/B676

Association of Dynamin-2 with Morphological Changes at Tight Junctions in Lung Alveolar Epithelial Cells.

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Acute respiratory distress syndrome (ARDS) is a severe and oftentimes fatal lung disease characterized by widespread fluid flooding of the lungs. Maintenance of fluid balance in the lung airspace is critical for survival of ARDS. Chronic alcohol abuse is associated with impaired fluid balance in the lungs and magnifies the severity and risk of ARDS. Our lab is currently investigating the molecular mechanisms of chronic alcohol abuse and the increased risk of ARDS with a particular interest in the effects on tight junctions. Previously we determined that an increase in tight junction protein claudin-5 was associated with chronic alcohol ingestion. This increased expression of claudin-5 was necessary and sufficient to reduce barrier function and induced the molecular rearrangement of tight junction proteins to form spike-like protrusions (referenced as tight junction spikes (TJ spikes)) perpendicular to the linear junction. Upon closer inspection of the TJ spike structures, we observed vesicle budding and fusion at the TJ spikes, suggesting that these structures could be areas of active junction remodeling, known to be facilitated by endocytosis of junction proteins. Upon treatment with endocytosis inhibitor Dynasore, which targets the actin-binding protein dynamin, we observed a decrease in the number of TJ spikes, suggesting a role for dynamin in TJ spike formation. We determined by immunoblot that dynamin-2 is the dominant isoform in primary rat lung alveolar type I epithelial cells and observed dynamin-2 localized to tight junctions and TJ spikes by immunofluorescence. Using super-resolution immunofluorescence microscopy (Stimulated Emission Depletion (STED)) to further investigate the localization of tight junction proteins within TJ spikes, we observed irregular distribution of ZO-1 compared to claudin-18 within the TJ spikes. We further observed several TJ spike intermediate structures that we hypothesize to be maturing TJ spikes. We are currently using live-cell microscopy and super-resolution microscopy to further characterize TJ spikes and their role in epithelial barrier function and maintenance. Our long-term goal is to identify novel therapeutic targets to rescue barrier function by restoring normal tight junction morphology.

Cadherins and Cell-Cell Interactions

P661/B677

Resolving the Mechanism of Adhesion Mediated by a Non-clustered Delta-1 Protocadherin.

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Cadherins form a large family of calcium-dependent cell adhesion proteins involved in cell differentiation, tissue morphogenesis and neuronal connectivity. Being membrane proteins, they have an extracellular, a transmembrane and a cytoplasmic domain. Non-clustered delta-1 protocadherins are a subgroup of cadherins with seven extracellular cadherin (EC) repeats, a single-pass transmembrane domain, and cytoplasmic domains distinct from classical cadherins. These proteins mediate homophilic adhesion and have been implicated in various diseases, including asthma, autism, epilepsy and cancer. However, how delta-1 protocadherins use their extracellular domain to mediate adhesion is unknown. Here we present the first X-ray crystal structures of the extracellular domain of a delta-1 protocadherin member, Protocadherin-1 (PCDH1), expressed mainly in the airway epithelium, skin keratinocytes, and lungs. PCDH1 is involved in asthma and hantavirus related pulmonary syndrome infection. The structures revealed binding modes that involve antiparallel as well as parallel overlap of multiple EC repeats. Mutagenesis, binding assays and other biochemical experiments tested the modes of adhesion as predicted from these structures. Overall, our studies reveal the molecular mechanisms of adhesion mediated by PCDH1 and shed light on its role in maintaining epithelial integrity, the loss of which causes asthma. Given the sequence similarity among delta-1 protocadherins, these mechanisms of adhesion are likely the mechanism of adhesion for all of them. Further, the structure of PCDH1 EC1, which is used for infection by Hantavirus will open up new avenues of drug design against the disease.

P662/B678

Resolving Species-dependent Heterophilic and Homophilic Cadherin Interactions in Intestinal Epithelium Intermicrovillar Links.

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Enterocytes are specialized epithelial cells lining the luminal surface of the small intestine involved in nutrient absorption, processing and host defense. Terminally differentiated enterocytes have densely packed arrays of microvilli known as the brush border. The microvilli are arranged in a hexagonal pattern that is maintained by intermicrovillar links formed by two non-classical members of the cadherin superfamily of calcium-dependent cell adhesion proteins: Protocadherin-24 (PCDH24) and the mucin protocadherin (CDHR5). Cadherins typically have an extracellular domain with a variable number of extracellular cadherin (EC) repeats followed by a transmembrane and a cytoplasmic domain. PCDH24 has 10 EC repeats and a membrane adjacent domain while CDHR5 has 4 EC repeats and membrane adjacent mucin-like domains. Intermicrovillar links are formed by the heterophilic and homophilic interactions of PCDH24 and CDHR5. Here we present binding assays that identify the PCDH24 and CDHR5 EC repeats involved in both heterophilic and homophilic adhesion for human and mouse proteins. We also present the first X-ray crystal structures of the PCDH24 and CDHR5 tips and analyze species-specific differences in the mode of adhesion of these proteins. Structures together with binding assays and mutagenesis are shedding light on how brush border assembly, key to nutrient absorption, is driven by cadherins.

P663/B679

Role of Jak/Stat Signaling during Neural Crest Migration.

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Many of the molecular mechanisms acting during early embryogenesis are reiterated during tumor initiation, progression and metastasis. The JAK/STAT pathway has a well established role in cancer cell invasion and collective cell migration in the Drosophila. Here, we show that JAK/STAT signaling also plays a role in neural crest migration during vertebrate development. Loss and gain of STAT3 function leads to defects in neural crest delamination and migration, at least partially due to dysregulation of key EMT transcription factors like Ets1 as well as the cell adhesion molecule E-cadherin. Our findings reveal a novel role for JAK/STAT signaling pathway during neural crest migration and suggest that is plays an evolutionarily conserved role in collective cell migration.

P664/B680

Kinetic Nucleation-dependent E-cadherin Clustering Regulates Alpha-Catenin Conformational Activation.

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E-cadherin-based cell-cell adhesion (E-cadherin adhesion) is essential for organogenesis and maintenance of the epithelial tissue, and a loss of cell- cell adhesion has been suggested to contribute to cancer development. In addition to performing its adhesive function through the extracellular domain-mediated homotypic interaction, E-cadherin adhesion enables cells to sense mechanical tension in the tissue through the adaptor protein, alpha-catenin. This has been proposed to be mediated through, in part, a conformational change in alpha-catenin upon increase in the actomyosin tension on E-cadherin adhesion resulting in the availability of cryptic binding sites in alpha-catenin leading to an enhancement in its interaction with vinculin, an adaptor protein that is homologous to alpha-catenin, and the actin cytoskeleton. Reconstitution of E-cadherin adhesion in a hybrid format wherein a live cell interacts with a synthetic, supported lipid bilayer functionalized with the extracellular domain of E-cadherin showed that cells utilize filopodia to cluster E-cadherin. Importantly, E-cadherin clustering requires a reduced diffusive mobility of the protein on the membrane indicating the presence of a step of kinetic nucleation in the process. Further, experiments with nanopatterned supported lipid bilayer substrates revealed that the size of E-cadherin clusters regulate the conformational activation of alpha-catenin. Once activated, however, alpha-catenin is sustained in the active conformation even when actomyosin tension in the cell is reduced pharmacologically. Thus, membrane diffusion-regulated E-cadherin clustering impacts alpha-catenin conformational activation and mechanical signaling.

P665/B681

Cell Proliferation Controls Cell Shape Remodeling in Epithelia.

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Epithelia have distinct cellular architectures, which are maintained despite externally applied forces, wounding, and cell division or death. In turn, cell shape is thought to affect tissue function by controlling cell differentiation, and motility. Here we investigate cell shape changes in a model epithelial monolayer of MDCK cells. After the onset of confluence, we find that cells continue to divide and change shape

over time, eventually reaching a similar final state characterized by arrested dynamics and more hexagonal cell shapes. On substrates of different stiffness, monolayers form with different cell density, but have a similar relationship between cell shape and dynamics. This suggests that cell shape controls motility in confluent monolayers. We find that inhibiting different cytoskeletal regulators causes monolayers to arrest with elongated cell shapes. Interestingly, across a diverse set of inhibitor conditions, final cell shape is correlated with the rate of cell division. By measuring the shape change of individual cells during monolayer remodeling we demonstrate oriented cell division is insufficient to explain this relationship. We observe similar changes in the cell shape behavior when modifying the effective surface tension in an active vertex model. This suggests that changes in cell shape remodeling result from differences in cell mechanical properties. To understand the mechanism which couples cell mechanics and proliferation in monolayers we perturb several molecular pathways which regulate both the cytoskeleton and the cell cycle. Our results reveal an important connection between cell proliferation and cell mechanics which controls cell shape remodeling in epithelia.

P666/B682

Junctional Localization of Septin 2 Is Crucial for Integrity of Junctional Molecules in Endothelial Monolayer.

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It was previously demonstrated that Septin 2 is required to regulate barrier function of microvascular endothelial monolayer. Loss of Septin 2 protein altered organization of vascular endothelial (VE)-Cadherin, adherens junctional molecule as well as dynamic membrane protrusion at the cell-cell contact sites. On top of that, we recently found that localization of Septin 2 at the junctions is necessary to control integrity of junctional molecules. TNF-alpha treatment with microvascular endothelial cells sequestered Septin 2 to the cytoplasm from the junctions without the effect of protein level. Interestingly, TNF-alpha treatment revealed the same effect with loss of Septin 2 on various junctional molecules of adherens junctions (AJs) and tight junctions (TJs). Immunofluorescent staining analysis showed dramatic loss of integrity of AJs and TJs at the junctions. Next, in order to investigate necessity of junctional localization of Septin 2 we performed mutations on amino acid residues in polybasic motif of Septin 2 suggested to interact with $PIP_{2\ in}$ plasma membrane. Of note, overexpression of PIP_2binding mutants of Septin 2 showed accumulation of Septin 2 in the cytoplasm with decrease in junctional localization in microvascular endothelial cells.

P667/B683

Inside-out Regulation of Cadherin Adhesion.

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During morphogenesis and wound healing, cellular migration and rearrangements rely on tightly regulated changes in E-cadherin (Ecad) adhesion. Previous studies, carried out in a cell free context, show that Ecad ectodomains bind in two different conformations, strand-swap dimers and X-dimers, which have different adhesive properties. However, the molecular mechanism by which Ecad conformation and adhesion is regulated on cell surface is not understood. To address this critical gap,

we used live cell Atomic Force Microscopy (AFM) to correlate the structure and adhesion of Ecad ectodomains to their cytoskeletal linked states in Madin-Darby Canine Kidney (MDCK) cells engineered with different knockout/mutant versions of α-catenin and vinculin. Our results show that in parental MDCK cells, only a third of non-junctional Ecads are robustly bound to the actin cytoskeleton; the remaining Ecads only transiently associate with the cortical actin. Robust linkage of Ecad to the cytoskeleton requires both α-catenin and vinculin. Strikingly, we show that ectodomains of cytoskeleton bound Ecad adopt a strand-swap dimer conformation while disrupting cytoskeletal linkage traps Ecads in X-dimer conformation. Our data directly demonstrates that changes in Ecad ectodomain structure and adhesion are regulated from the inside-out, by Ecad linkage to the actin cytoskeleton.

P668/B684

Endothelial Junctional Tunnels Are Crucial for Leukocyte Extravasation.

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Inflammation is characterized by migrating leukocytes that extravasate from the circulation across the vascular endothelium into inflamed tissue. This is a carefully orchestrated dialogue of adhesion and signaling between leukocytes and endothelial cells. The process is tightly regulated to prevent excessive leukocyte extravasation and vascular leakage. In our study, we demonstrate the role of previously understudied structures at the endothelial level and their role in extravasation using a multitude of advanced imaging techniques (AFM, SEM, TEM, CLSM, and SIM). Using these techniques, we show that endothelial cell junctions are overlapping instead of connecting. To allow for better study of this process we adopted and developed an advanced microfluidic vessel-on-a-chip approach to grow vessels resembling postcapillary venules. These functional vessels are created in a modular system allowing versatility in matrix substance, stiffness, lumen size and cell type(s). We show that this overlapping structure exists in *vitro*, in our vessel on a chip and in *vivo*. Using mosaic expression of specialized membrane-targeting fluorescent constructs, we were able to demonstrate that these overlapping structures play a crucial role in neutrophil extravasation by the formation of tunnels that help neutrophils to crawl through during diapedesis. This work challenges the extravasation dogma stating that endothelial cells would transiently disconnect to allow leukocytes to pass.

P669/B685

Formation of Intercellular Nanotubes Via Helical Deformation.

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Extensive studies on intercellular nanotubes (INT) that physically connect cells have revealed their biological significances as a new pathway for the distant transport of cytoplasmic components, viruses, or pathogenic substances between cells. However, it remains unknown how such a fine structure is formed and sustained robust over long distance for several hours. Using real-time optical imaging in various epithelial cells, we visualized that random contact of filopodia protruding from two different cells forms filopodial bridges (FBs) via cadherin-cadherin interactions and one of the filopodia is ultimately released from the other filopodium, which result in a single long filopodium bridge connecting two cells (INT). Interestingly, super-resolution microscopy revealed a fine helical structure of FBs, which is driven by rotational motions of filopodia due to the twist of actin filaments inside filopodia by myosin-V. Mechanical studies and computer-aided simulation strongly suggest that the transition of FB into INT is likely that accumulated torsional energy in FBs with the helical deformation breaks

cadherin-cadherin interactions between two filopodia and the actin retrograde flow of the released filopodium leaves a single bridge attached to a paired cell. The single bride is strongly supported by cadherin-cadherin clusters at the junction between the end of the bridge and the cell, which is likely a synapse. This study sheds light on the formation mechanism of filopodial bridge-based intercellular nanotubes for long-distance communication between cells.

P670/B686

Expression Profile of Type I and Type II Classical Cadherins during Neuronal Nuclei Formation in the Developing Mouse Subpallium.

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The Differential Cell Adhesion Hypothesis of tissue separation states that cell sorting results from differences in intercellular adhesiveness determined by cell adhesion molecules (CAMs) expressed on the cell surface. Type I and type II classical cadherins comprise a group of CAMs that regulate cell sorting by maximizing adhesive strength mediated by homo and heterotypic cadherin binding. Factors that affect the ability of cadherins to regulate cell sorting include the affinity of cadherin binding and their expression levels. This study examines the expression of cadherins type I (CDH1, CDH2, CDH3, CDH4, and CDH15), type II (CDH5, CDH6, CDH7, CDH8, CDH9, CDH10, CDH11, CDH12, CDH18, CDH19, CDH20, CDH22 and CDH24), and the CELSR CDH13 during nuclei formation in the developing mouse subpallium. Cadherin's mRNA expression values were obtained from in *situ* hybridization experiments carried out at the Allen Brain Institute and available at the Allen Developing Mouse Brain Atlas (DMBA). Expression values for each structure were calculated using the Structure Unionizer module and expression energy values ((sum of expressing pixel intensity / sum of expressing pixels) * (sum of expressing pixels / sum of all pixels)) for each structure were analyzed at embryonic (E) 13.5, E15.5, postnatal (P) 28 and P56. At P56, all type I and type II cadherins are detected in the subpallium; however, only CDH2, 8, 11 and 13 show high expression values (between 1 and 16) throughout the subpallium, while CDH5, 6, 9, 10, 12, 18, 20 and 24 show expression values above 1 in only one to three substructures, and CDH1, 3, 4 and 7 show very low expression (expression values for CDH15, 19 and 22 are not available at the DMBA). Expression values at the ontological structure level 8 (which includes twelves substructures) show a progressive increase in expression of CDH2, 8, 11, and 13 throughout development with regional differences in cadherin type and expression level. CDH5, 6, 9, 10, 12, 18, 20 and 24 show variable expression patterns at different stages. The Pearson's correlation coefficient calculated at each developmental stage between cadherins with the highest expression values (CDH2, 8, 11 and 13) show that expression of CDH2 and 13 is significantly correlated at E13.5 ($r = 0.529$), P28 ($r = 0.689$) and P56 ($r = 0.616$), expression of CDH11 and 13 is correlated at E13.5 ($r = 0.569$), while CDH8 and 13 expression is correlated at P28 ($r=0.689$). This analysis shows that cadherin type and expression level are heterogenous among cell pools within the subpallium. This suggest that groups of cells within the subpallial mass have distinct and dynamic patterns of type I and II cadherin expression that may provide an identity that contributes to the segregation of cells from a parental cell group and their aggregation into distinct nuclei.

P671/B687

αT-catenin Intramolecular Interactions Regulate Vinculin Binding.

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Cardiomyocyte contractions place unique physical and regulatory demands on the protein complexes that join these cells. Cardiomyocytes are connected by intercalated discs (ICDs), specialized junctions that mechanically and electrically couple cells. Mechanical linkage at the ICD is accomplished through the adherens junction (AJ) and desmosome that connect to the actin and intermediate filament cytoskeletons, respectively. The core component of the AJ is the cadherin-catenin complex, and α-catenin serves as the primary link between the AJ and actin. Two α-catenins are expressed in the mammalian heart, αE(Epithelial)-catenin and αT(Testes)-catenin. Tension regulates αE-catenin conformation: actomyosin-generated force stretches the middle(M)-region to relieve autoinhibition and reveal a binding site for the actin-binding protein vinculin. In contrast, little is known about the molecular properties of αT-catenin. Here we describe the biochemical properties of αT-catenin and how intramolecular interactions regulate vinculin binding. Isothermal titration calorimetry (ITC) showed that αT-catenin binds the β-catenin/N-cadherin complex with a similar affinity (low nanomolar) to that of αE-catenin indicating a similar association with the AJ. Limited proteolysis revealed that the αT-catenin M-region adopts a more open conformation than αE-catenin. Consistent with these results, ITC revealed that the αT-catenin M-region binds the vinculin N-terminus with low nanomolar affinity, indicating that the αT-catenin M-region is not autoinhibited and distinct from αE-catenin. However, the αT-catenin head domain binds vinculin 1000-fold more weakly (low micromolar affinity), indicating that the N-terminus regulates M-region binding to vinculin. Notably, β-catenin binding to the αT-catenin head domain does not affect vinculin binding. Together, our results indicate that the αT-catenin N-terminus is required to maintain M-region autoinhibition and modulate vinculin binding. We postulate that the unique molecular properties of αT-catenin regulate ligand binding to meet the demands of intercellular adhesion in cardiomyocytes.

P672/B688

Epithelial Cells Act as a Regional Checkpoint for Immune Cell Organization.

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Skin protects our body against the outer environment, and its ability to repair upon injury is directly connected to both disease and survival. Despite recent advances in our understanding of skin homeostasis, it is still unclear how stem cells interact with their niche to sustain this process in a live mammal. The critical barrier to addressing these fundamental questions lies in the inability to simultaneously follow behaviors of different cell types and to define their functional interactions in *vivo*. Skin epidermis is an ideal system because of its accessibility and well-characterized epithelial stem cells and coexisting epidermal immune cells. In the epidermis, epithelial stem cells are closely intermingled with two resident immune cell populations: Dendritic epidermal T cells (DETCs) and Langerhans cells (LCs). Intravital imaging platform with multiphoton microscope allows us to dissect the coordination and functional significance of distinct cell activities, populations and interactions during homeostasis in live mice. In addition to immune surveillance, our data show that both immune populations can perceive and respond to the changes of their neighbors. Epidermal immune cells actively retain spatial organization within their own population while neighboring epithelial stem cells continuously divide and

differentiate. Furthermore, skin epithelial stem cells act as regional checkpoints for the organization and number of epidermal immune populations, but not vice versa. This study reveals new principles of immune organization within the epidermis and elucidates dynamic epithelial-immune interactions that are in place to maintain homeostasis of the epidermis.

P673/B689

RhoA mediates epithelial cell shape changes via mechanosensitive endocytosis.

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RhoA mediates epithelial cell shape changes via mechanosensitive endocytosis Kate E. Cavanaugh[1,2], Michael F. Staddon[3], Ed Munro[1,4], Shiladitya Banerjee[3], Margaret L. Gardel[4, 5 1] Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL 60637, USA, [2]Committee on Development, Regeneration and Stem Cell Biology, University of Chicago, Chicago, IL 60637, USA, [3]Department of Physics and Astronomy, and Institute for the Physics of Living Systems, University College London, London WC1E 6BT, United Kingdom, [4]Institute for Biophysical Dynamics, University of Chicago, Chicago 60637 IL, USA, [5]James Franck Institute, and Department of Physics, University of Chicago, Chicago 60637 IL, USA. Abstract: Epithelial remodeling involves ratcheting behavior whereby periodic contractility produces transient changes in cell-cell contact lengths, which stabilize to produce lasting morphogenetic changes. Pulsatile RhoA activity is thought to underlie many morphogenetic ratchets, but how RhoA governs transient changes in junction length, and how these changes are rectified to produce irreversible deformation, remains poorly understood. Here, we use optogenetics to characterize responses to pulsatile RhoA in model epithelium. Short RhoA pulses drive reversible junction contractions, while longer pulses produce irreversible junction length changes that saturate with prolonged pulse durations. Using an enhanced vertex model, we show that this is explained by two effects: thresholded tension remodeling and continuous strain relaxation. Our model predicts that structuring RhoA into multiple pulses overcomes the saturation of contractility and we confirm this experimentally. Junction remodeling also requires formin-mediated E-cadherin clustering and dynamin-dependent endocytosis. Thus, irreversible junction deformations are regulated by RhoA-mediated contractility, membrane trafficking, and adhesion receptor remodeling.

P674/B690

PI3K Regulates Adhesions in Parietal Endoderm Migration.

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Parietal endoderm outgrowths from F9-derived Embryoid Bodies (EB) are a well-established model system for the in *vitro* study of morphological and molecular events involved in early embryonic yolk sac development including migration. Migration of parietal endoderm mimics a combination of collective sheet migration and contact inhibition of locomotion in an oriented and directed fashion away from the embryoid body. Data suggest that these transient cell to cell interactions are comprised of classical cadherin family adhesion proteins typical of adherens junctions, while cell-ECM interactions are

mediated by typical integrin-containing focal adhesions. Recently, our laboratory has begun to investigate the role of Phosphatidylinositol 3-Kinase (PI3K) signaling in parietal endoderm migration. Inhibition of PI3K using LY294002 phenocopies the effects of siRNA knockdown of E-cadherin. Preliminary data suggest that inhibition of PI3K results in a decrease in E-cadherin containing adhesions at cell-cell boundaries, with a corresponding increase in vinculin containing focal adhesions within the sheet of outgrowth. Vinculin containing focal adhesions on the leading edge of the outgrowth appear to be more numerous and robust. These data suggest a role for PI3K in regulating cell-cell and cell-ECM adhesions during the migration of parietal endoderm Here, we explore the relationship between PI3K and E-cadherin during parietal endoderm migration.

P675/B691

Regulating Interactions between Cell-cell Adhesion Proteins P120-catenin and Plekha7.

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The adherens junction (AJ) is a crucial mediator of cell-cell adhesion and promotes the integrity and stability of epithelial tissue. Additionally, the AJ has important roles in regulating cell signaling, promoting the formation of other junctional complexes, maintaining cytoskeletal integrity, transducing mechanical inputs, and others. The AJ is composed primarily of a core cadherin-catenin complex, including E-cadherin, p120-catenin, β-catenin, and α-catenin. This core cadherin-catenin complex localizes along basolateral contacts and also exhibits a highly organized concentration at the apical membrane. Our lab recently showed the functional relevance of these two distinct cadherin-catenin populations. The apical complex, characterized by the presence of PLEKHA7 (P7), suppresses pro-anchorage independent growth factors, which are driven by the basolateral complex. This regulatory action of the apical complex is dependent upon the presence of P7. P7 recruits components of the RNA-interference pathway, including microRNA processing complex, RNA-induced silencing complex, and a subset of miRNAs and mRNAs, to the apical AJ and thereby mediates localized regulation of translation. The loss of P7 leads to upregulation of pro-anchorage independent growth and pro-tumorigenic proteins, including Snail, Myc and Cyclin D1. It is known that P7 is localized exclusively to the apical AJ through its interaction with p120-catenin. However, nothing is known about how this interaction is regulated. Here, we explore the regulation of the P7-p120-catenin interaction by phosphorylation. We characterize the small binding domain of p120-catenin required for the interaction. Finally, we begin to explore functional effects of this regulation at the AJ and potential implications for normal physiology and disease states.

P676/B692

Investigating the Interactome of the Brush Border Protocadherin Cdhr5 in Transporting Epithelia.

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Transporting epithelia of the intestine and kidney mediate solute transport by assembling a brush border (BB): a highly-ordered array of specialized apical microvilli. BB assembly is driven, in part, by a protocadherin-based adhesion complex found at the tips of BB microvilli. Known as the intermicrovillar adhesion complex (IMAC), this protein complex creates physical linkages that connect neighboring microvilli together during BB assembly in order to establish a mature, functional BB. IMAC adhesion is mediated by two calcium-dependent protocadherin molecules: Cadherin Related Family Member 2 (CDHR2; also known as protocadherin-24) and Cadherin Related Family Member 5 (CDHR5; also known

as mucin-like protocadherin). Previous studies revealed that CDHR2 is associated with a cytoplasmic complex comprised of two scaffolding proteins, USH1C and ANKS4B, along with the motor protein Myo7b. To further define the protein composition of the IMAC, we performed a screen to isolate proteins that associate with the cytoplasmic domain of CDHR5 and have identified the PDZ-based scaffold E3KARP as a putative binding partner. Importantly, E3KARP and its closely related homolog EBP50 are highly expressed in the transporting epithelia of the kidney and small intestine, respectively. Here, these scaffolds are thought to associate with Ezrin and numerous transmembrane proteins in order to promote both proper microvillar structure and function. E3KARP/EBP50 are each comprised of two PDZ domains followed by an Ezrin binding domain that terminates with a canonical PDZ-binding motif. Given that EBP50 has been shown to play an essential role in intestinal BB assembly, we focused our attention on characterizing this candidate binding partner using our intestinal models. We observe that CDHR5, EBP50 and Ezrin exhibit partial co-localization within BB microvilli, and that the phosphorylated, active form of Ezrin displays an enrichment towards the tips of BB microvilli, the site of IMAC function. Furthermore, we provide preliminary biochemical data to demonstrate that ternary complex formation between CDHR5-EBP50-Ezrin requires Ezrin to be in an active conformation. Going forward, we will investigate whether sequential activation of Ezrin by $PI(4,5)P_2$ binding and phosphorylation leads to CDHR5-EBP50-Ezrin ternary complex formation. In sum, these results further define the IMAC interactome and reveal a potential mechanism of how CDHR5 may contribute to BB structure through Ezrin.

P677/B693

Investigating the Function and Regulation of the Apical Scaffold an ks4b during Enterocyte Brush Border Assembly.

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The transporting epithelia of the intestine interact with their luminal environment by assembling an apical brush border: a densely-packed collection of lumen-oriented microvilli that are highly enriched in nutrient processing enzymes and transport channels. Although essential in gut function, how enterocytes assemble a functional brush border is still poorly understood. Recent studies have shown that a protocadherin-dependent adhesion complex is a key factor in driving brush border assembly. Microvillar organization is mediated by a pair of protocadherins (CDHR2 and CDHR5) that interact in *trans* to create physical linkages between the neighboring microvilli at their distal tips. To permit correct tip targeting and function, these protocadherins require association with a cytoplasmic complex that includes two scaffolding proteins, Harmonin and ANKS4B, and a myosin motor, Myo7b. Altogether, these components are known as the intermicrovillar adhesion complex (IMAC). Here, we provide evidence that ANKS4B acts as the 'primordial' scaffold used to assemble the IMAC within the enterocyte apical domain in a regulated manner. ANKS4B is comprised of an N-terminal ankyrin-repeat domain, a central unstructured region and C-terminal sterile α-motif ending in a PDZ-binding motif. While ANKS4B has been shown function as a scaffold by directly interacting with Myosin-7b and Harmonin, little is known about either its regulation or targeting properties. We show that ANKS4B is the first IMAC component expressed during enterocyte polarization, and that ANKS4B possesses a dedicated BB-targeting domain that promotes apical targeting independent of the other adhesion complex components. Furthermore, we demonstrate that ANKS4B is subject to phosphorylation, and that phosphorylation of the scaffold modulates its functional properties during BB assembly. Together, our

study further defines the role of ANKS4B within the IMAC and sheds light on how dysfunction of ANKS4B results in perturbed BB structure, which may contribute to GI disease.

Cell Death: ROS & ER-Stress

P678/B695

Haploinsufficiency of U17 Small Nucleolar Rnas Reprograms Cellular Metabolism and Regulates Metabolic Stress.

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Dyslipidemia and resulting lipotoxicity are common features of metabolic syndrome and type 2 diabetes. Excess lipid causes cell dysfunction and induces cell death through pleiotropic mechanisms that link to oxidative stress. Through a retroviral promoter trap mutagenesis screen, we isolated a cell line that is resistant to lipid-induced cell death and oxidative stress. We show that disruption of the box H/ACA U17 snoRNAs encoded within the small nucleolar hosting gene 3 *(Snhg3*) causes resistance to lipid-induced cell death and general oxidative stress. This protection from metabolic stress results from upregulation of the mammalian target of rapamycin (mTOR) signaling axis and is associated with increased oxidative metabolism. Our findings demonstrate a novel role for the U17 snoRNAs as regulators of cell metabolism and stress response pathways.

P679/B696

MUC5B Expression in IPF is Associated with ER Stress and Impaired Mitochondrial Function.

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Rationale: Idiopathic Pulmonary Fibrosis (IPF) is a chronic progressive lung disease characterized by parenchymal remodeling and heterogeneous fibrosis due to poorly understood mechanisms. The gain-of-function *MUC5B* promoter variant rs35705950 is the strongest risk factor for IPF. MUC5B protein expression is upregulated in bronchoalveolar epithelial cells and honeycomb cysts in patients with IPF. Pulmonary fibrosis develops as a final pathological outcome of impaired wound healing responses to persistent lung injury and is characterized by the excessive extracellular matrix (ECM) deposition in the lungs. It is known that persistent endoplasmic reticulum (ER) stress in alveolar epithelial cells is observed in IPF. CHOP (a marker of ER stress) is often activated in the terminal or pathological stage to induce activation of Caspase 3 usually leading to apoptosis. Thus, we hypothesize that overproduction of MUC5B in bronchoalveolar epithelia is associated with the ER stress response leading to cell death and progressive lung fibrosis. **Methods and Results:** Using RNAScope and immunofluorescence, we have found that different cell types express MUC5B, including a subset of type II epithelial cells in human lung tissues obtained from controls and patients with IPF. MUC5B has higher expression in IPF lungs than control lungs, and is strongly associated with honeycomb cysts, where we also found majority of CCSP-positive and a subset of SPC-positive cells producing MUC5B. Moreover, MUC5B is co-expressed with CHOP in airway epithelial cells as well as in a subset of SPC-positive cells in patients with IPF. Some MUC5B overexpressing cells in IPF patients co-express cleaved caspase 3, indicating mitochondrial damage. SHG for fibrillar collagen analysis showed increased fibrillar collagens depositions in patients with IPF. Local metabolic imaging via Fluorescent Lifetime Imaging Microscopy (FLIM) showed that patients with IPF have a shift of free/bound NADH lifetime towards increased free NADH in airway

epithelial cells and alveolar regions, indicating metabolic reprogramming of airway cells consistent with mitochondrial damage and thus impaired function. **Conclusions:** These results indicate that excess of MUC5B is associated with ER stress in the distal lung, which may cause mitochondrial impairment and apoptosis, followed by augmented lung fibrosis.

P680/B697

L-serine Protects Mouse Hippocampal Neuronal Ht22 Cells Against Oxidative Stress-mediated Mitochondrial Damage and Apoptotic Cell Death.

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The cytoprotective mechanism of L-serine against oxidative stress-mediated neuronal apoptosis was investigated in mouse hippocampal neuronal HT22 cells. Treatment with the reactive oxygen species (ROS) inducer 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) increased cytosolic and mitochondrial ROS and apoptosis, without necrosis, in HT22 cells. ROS-mediated apoptosis was accompanied by the induction of the endoplasmic reticulum (ER) stress-mediated apoptotic pathway, involving CHOP/GADD153 upregulation, JNK and p38 MAPK activation, and caspase-12 and caspase-8 activation, and subsequent induction of the mitochondrial apoptotic pathway through BAK and BAX activation, mitochondrial membrane potential (Δψm) loss, caspase-9 and caspase-3 activation, PARP cleavage, and nucleosomal DNA fragmentation. However, the DMNQ-caused ROS elevation and ER stress- and mitochondrial damage-induced apoptotic events were dose-dependently suppressed by co-treatment with L-serine (7.5−20 mM). Although DMNQ reduced both the intracellular glutathione (GSH) level and the ratios of reduced GSH to oxidized GSH (GSSG), the reduction was restored by co-treatment with L-serine. Co-treatment with GSH or *N*-acetylcysteine also blocked DMNQ-caused ROS elevation and apoptosis; however, co-treatment with the GSH synthesis inhibitor buthionine sulfoximine significantly promoted ROS-mediated apoptosis and counteracted the protection by L-serine. In HT22 cells, DMNQ treatment appeared to tilt the mitochondrial fusion-fission balance toward fission by down-regulating the levels of profusion proteins (MFN1/2 and OPA1) and inhibitory phosphorylation of profission protein DRP1 at Ser-637, resulting in mitochondrial fragmentation. These DMNQ-caused alterations were prevented by L-serine. A comparison of mitochondrial energetic function between DMNQ- and DMNQ/L-serine-treated HT22 cells showed that the DMNQ-caused impairment of the mitochondrial energy generation capacity was restored by L-serine. These results demonstrate that L-serine can protect neuronal cells against oxidative stress-mediated apoptotic cell death by contributing to intracellular GSH synthesis and maintaining the mitochondrial fusion-fission balance.

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Genome-wide Crispri and Crispra Screens Identify Novel Factors Regulating Reactive Oxygen Species (ros) Homeostasis in Human Ipsc-derived Neurons.

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Reactive oxygen species (ROS) are highly reactive oxygen-derived radicals that can damage proteins, lipids, DNA and RNA. Excessive level of ROS has been implicated in aging and many neurodegenerative diseases. Neurons in the brain are especially vulnerable to ROS due to their high oxygen consumption, limited antioxidants and enriched polyunsaturated fatty acids (PUFA) that are susceptible to oxidation. However, how neurons maintain ROS homeostasis remains largely unknown. To systematically characterize factors that regulate ROS level in human neurons, we conducted multiple genome-wide CRISPR-based genetic screens. We first developed platforms that enable highly specific gene knockdown or overexpression in human neurons by integrating CRISPRi/a technologies with our previously established i^{3}N neuronal differentiation method that produce large amounts of homogenous glutamatergic neurons. Next, we conducted genome-wide loss-of- and gain-of-function screens in those neurons, including reporter-based screens on the level of ROS, and survival-based screens, in which neurons were cultured in normal medium or medium lack of antioxidants thus generating a condition of mild oxidative stress. From these screens, we identified novel genes that regulate ROS level or neuronal survival under oxidative stress. One of the top hits, prosaposin (encoded by gene *PSAP*), a lysosomal protein involved in gangliosides degradation, showed a strong increase of ROS level when knocked down in neurons. We further characterized that loss of *PSAP* leads to ganglisoides accumulation in enlarged lysosomes, enhanced autophagy and damaged mitochondria in a neuron-specific manner. *PSAP* knockout neurons are highly susceptible to the medium lacking antioxidants, which can be fully rescued by inhibiting lipid peroxidation by Ferrostatin-1 or iron chelation by Desferoxamine (DFO), but not by caspase inhibition, suggesting that ferroptosis but not apoptosis is induced in *PSAP* knockout neurons under oxidative stress. Furthermore, we identified ferroptosis signatures including dramatic accumulations of lipid peroxidation and iron, and decreased glutathione (GSH) level in *PSAP* knockout neurons. In summary, we developed CRISPRi and CRISPRa screening platforms in human iPSC-derived neurons and uncovered novel factors that regulate ROS level in human neurons through genome-wide CRISPR screens. Among all the novel hits, we elucidated the mechanism of *PSAP,* which provides novel links between gangliosides metabolisms, autophagy and ferroptosis.

P682/B699

A Small Molecule Piperazine Oxalate Derivative Compound Induces Er Stress-mediated Apoptosis through Activation of the Activating Transcription Factor 4-c/ebp Homologous Protein-death Receptor 5 Pathway.

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Modulation of unfolded protein response (UPR) is an emerging target pathway to treat cancer for its cell death induction activity. In our procedure to isolate UPR-modulating small molecules, we previously reported a piperazine oxalate derivative compound (AMC-04) triggering enhanced phosphorylation of eukaryotic translation initiation factor-2 alpha (eIF2α). An d in this report, we found that AMC-04 induces apoptotic cell death *via* activation of UPR in human hepatocellular carcinoma and human breast cancer cells. Incubation of breast cancer cells with AMC-04 induces upregulation of activation transcription factor-4 (ATF4)-C/EBP homologous protein (CHOP) followed by death receptor 5 (DR5) expression which was revealed by microarray analysis, small-interference RNA analysis, and western blotting. In more mechanistic view, AMC-04 induced cytotoxic UPR pathway activation is mediated by reactive oxygen species (ROS) generation. Chemical informatics approach predicted that AMC-04 can bind and modulate activity of histone methyltransferases. Furthermore, chemical or genetic inhibition of predicted target proteins induced UPR activation with resulting apoptotic cell death suggesting inhibition of histone methyltransferase are promising targets for future cancer therapy. Taken together, we identified a new small molecule AMC-04 modulating epigenetic enzyme activity, and AMC-04 revealed a new molecular communication between UPR and histone modification.

P683/B700

Cellular Energy Stress Induces ROS-mediated Activation of RIP3.

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The receptor-interacting serine-threonine kinase 3 (RIP3) is key molecule in the programmed necrotic cell death pathway. In response to TNF stimulation, RIP3 interacts with RIP1 through a homotypic interaction motif (RHIM) and MLKL is recruited to assemble necrosome. This consequently induce necroptosis. Phosphorylation is one of the post-translational modification and plays role of switch turning signal on/off. RIP3 is serine/threonine kinase and it can be activated by autophosphorylation at Ser 227 residue under TSZ induced necroptosis. Since phosphorylated RIP3 is required to bind MLKL, activation of RIP3 is critical to induce necrotic cell death. To date, however, how phosphorylation of RIP3 is regulated has not been known yet. Almost cancer cell is exposed to energy stress condition. Under cellular energy stress condition, we observed the phosphorylation of RIP3. Also protein A, which is one of the candidate for kinase to be activation under energy stress condition, phosphorylates RIP3 and sequentially activates MLKL. Furthermore though RIP1 is indispensable in TNF induced necroptosis pathway, it is not required in this condition to activate RIP3 and MLKL. In a future study we investigate protein a as the one of the candidates to activate RIP3.

P684/B701

Cell Injury Leads to an Immediate Internal Calcium Release in Surrounding Astrocytes.

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Astrocytes in the brain are rapidly recruited to sites of injury where they enclose the lesion, phagocytose damaged material and take up neurotransmitters and ions to avoid the propagation of damaging molecules. Excitatory molecules released from dying cells can lead to calcium dysregulation. Excessive calcium can cause mitochondrial depolarization and overload which can lead to necrotic cell death. We investigate the astrocytic response to nearby cell injury by utilizing a laser microscope system to induce cell death on a selected cell in an established astrocyte cell line. Our results show that astrocytes exhibit an immediate calcium spike. We hypothesize that the calcium spike in nearby cells is due to an internal release and influx from the outside. Glutamate is one of the excitatory molecules that is released from dying cells and can lead to a calcium influx through it's interaction with the NMDA receptor. We find that the NMDA antagonist MK801 decreases the intracellular calcium spike caused by a nearby cell injury. We also investigate the contribution of internal calcium release to the cytoplasmic spike through the use of a commercially available calcium free medium. Surprisingly, cells exhibited a calcium spike with greater amplitudes when compared to control cells in regular medium. Similarly, cells in calcium free hanks buffer saline solution are capable of exhibiting a calcium spike. Treatment with the Ip3 receptor antagonist and store operated calcium release modulator, 2-Aminoethoxydiphenylborane(2-APB) led to a decrease in the calcium spike when in regular and calcium free medium. These results support the internal release of calcium from the ER in astrocytes responding to injury or death in nearby cells.

P685/B702

Evaluation of Ph as a Factor in Vaam-mediated Cellular Distress and Death in *Euglena*.

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During the process of cellular respiration, cells generate a proton motive force (PMF). The PMF functions as a result of the electrochemical gradient found across the mitochondria's inner membrane, moving protons and electrons through a series of electron carrier proteins that, ultimately, provide power to ATP synthase. We observed Vespa Amino Acid Mixture (VAAM), a commercially available sports drink derived from Asian giant hornet larvae. VAAM is a suspected extreme coupler of the PMF and our previous studies have demonstrated increased production of ATP and reactive oxygen species under VAAM treatment, and death in both cells and invertebrates treated with VAAM. We are interested to see if VAAM is toxic to cells due to mitochondrial membrane disruption or due to the acidity of VAAM. VAAM suspended in tap water (as for *Euglena* incubation) has a pH of 3.6 at 0.6%, 3.6 at 0.3%, and 5.6 at 0.03% concentrations. *Euglena* were tested for survivability in the presence of VAAM, citric acid, and hydrochloric acid (HCl) at three different concentrations. *Euglena* have been shown to break down their flagella and enter a balled state when in toxic environments, this has been noted as cellular distress, and disrupted membranes have been noted as cellular death. We found that at 0.6%, 0.3%, and 0.03% VAAM *Euglena* cells show distress and cellular death compared to pH matched solutions of citric acid and HCl, which when tested did not show cellular distress or death. Death was expected to increase over time during the assay, so each trial (n = 3/treatment) was observed at 5, 15, and 30-minute intervals for signs of cellular death. It was found that *Euglena* showed increased cellular distress in response to

VAAM at 30 minutes relative to the 5- and 15-minute intervals. We conclude that while the amino acid mixture in VAAM does alter pH, pH is not sufficient to cause cell distress and death in the absence of VAAM.

P686/B703

Bap31 Inhibits Cell Adaptation to Er Stress Conditions, Negatively Regulating Autophagy Induction by Interaction with Stx17.

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Cancer cells modulate their metabolism to proliferate and survive under nutrient limitation and anaerobic stress conditions, and to induce cellular reactions such as unfolding protein response (UPR) and autophagy. UPR maintains and restores endoplasmic reticulum (ER) homeostasis to increase protein secretion by inducing the ER chaperons that mediate protein refolding and degrade unfolded proteins. However, irreversible ER stress induces cell death to eliminate damaged cells. Autophagy can play a crucial role in cellular adaptation to starvation. By recycling metabolites, autophagy provides a source of energy and basic factors for the biosynthesis of new macromolecules. Therefore, resistance to ER stress and the induction of autophagy is essential for tumor proliferation and survival, and the suppression of these processes is an important “metabolic checkpoint” for the elimination of cancerous cells; however, the molecular mechanism by which cancerous cells avoid the metabolic checkpoint is not fully understood. B-cell receptor associated protein 31 (BAP31) is an integral ER membrane protein that functions in a number of apoptotic pathways. Recently, we discovered that BAP31 regulates mitochondrial function and metabolic pathways, such as autophagy, by communication with ER and mitochondria via contact sites; however, the role of BAP31 in the metabolic checkpoint and cancerous phenotype is not fully understood. This study reports that BAP31 inhibits resistance to metabolic stress in cancer cells, induces cell death via ER stress, and acts as a tumor suppressor factor by suppressing autophagy via the metabolic checkpoint. The loss of BAP31 increased autophagy and suppressed ER stress-induced cell death. The autophagy related protein (ATG) family and the ER-resident SNARE protein syntaxin 17 (STX17) are well-known factors in the induction of autophagy signaling. BAP31 interacts with STX17, which suppressed the induction of autophagy by suppressing interaction with STX17 and ATG14L. Moreover, BAP31-knockout enhances invasion activity and tumor growth in metabolic stress conditions *in vivo* compared to BAP31 expressing cells. Therefore, BAP31 can be considered a novel tumor suppressor factor involved with metabolic stress, inducing cell death via the ER stress response and suppressing autophagy by inhibiting STX17 and ATG14L interaction.

P687/B704

A CRISPR Screen Identifies Essential Mediators of Hydrogen Peroxide-induced Cell Death.

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Hydrogen peroxide (H_2O_2) is a key regulatory molecule in cellular redox biology. When present at homeostatic concentrations, H_2O_2 can act as a signaling molecule for cell survival, proliferation and differentiation. However, when present at pathologically higher concentrations, H_2O_2 can cause oxidative stress which results in the damaging of proteins, lipids and DNA. This oxidative damage lead to cell death if the stress is not alleviated. Genes that are required for the execution of H_2O_2-induced cell death are currently unknown. Consequently, we conducted a comprehensive CRISPR-based positive selection screen to identify genes that are essential for H_2O_2-induced cell death in HAP1 cells. Our screen

revealed several genes that are involved in the generation of reactive oxygen species as necessary mediators of H_2O_2-induced cell death. Since H_2O_2has important implications for diseases that arise from oxidative stress such as cancer, myocardial infarction, and neurodegenerative diseases, our findings not only provide important insights on redox biology, but they may also inform avenues for therapeutic interventions for some of these diseases.

Regulation of Aging

P688/B705

Prmt7 Methylates and Suppresses Gli2 Binding to Sufu Thereby Promoting Its Activation.

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Cellular senescence is implicated in aging or age-related diseases. Sonic hedgehog (Shh) signaling, an inducer of embryonic development, has recently been demonstrated to inhibit cellular senescence. However, the detailed mechanisms to activate Shh signaling to prevent senescence is not well understood. Here, we demonstrate that Protein arginine methyltransferase 7 (PRMT7) promotes Shh signaling via GLI2 methylation which is critical for suppression of cellular senescence. PRMT7-deficient mouse embryonic fibroblasts (MEFs) exhibited a premature cellular senescence with accompanied increase in the cell cycle inhibitors p16 and p21. PRMT7 depletion results in reduced Shh signaling activity in MEFs while PRMT7 overexpression enhances GLI2-reporter activities that are sensitive to methylation inhibition. PRMT7 interacts with and methylates GLI2 on arginine residues 225 and 227 nearby a binding region of SUFU, a negative regulator of GLI2. This methylation interferes with GLI2-SUFU binding, leading to facilitation of GLI2 nuclear accumulation and Shh signaling. Taken together, these data suggest that PRMT7 induces GLI2 methylation, reducing its binding to SUFU and increasing Shh signaling, ultimately leading to prevention of cellular senescence.

P689/B706

Shh Signaling Regulates Cell Structure and Terminal Differentiation of Mouse Intervertebral Disc Cells.

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Sonic hedgehog (SHH) expressed by the notochord is a crucial regulator of embryogenesis. Using a mouse model system, we showed that the notochord descendant nucleus pulposus cells (NP) continue to express SHH in the center of each intervertebral disc (IVD), and throughout the spine. IVD degeneration is a major cause of back pain, but with no effective treatments, due to poor knowledge about the molecular and cellular processes that regulate healthy IVD. We also showed that SHH continues to be an important signaling molecule and regulate the growth, differentiation by way of extracellular matrix production, and structure of the neonatal mouse disc. Recently we found that with aging the expression of SHH reduced, which is associated with the differentiation of large uninucleated NP cells into small-multinucleated syncytium as shown by lineage-tracing studies. In the current study, we test the hypothesis that SHH is crucial for the maintenance of NP cells structure and function and whether it can regulate cellular plasticity to prevent or revert the pathological multinucleated phenotype of the NP cells. First using Shh reporter mice, we found that although SHH was expressed by all notochordal cells, in adult mouse, SHH is expressed only by a subset of NP cells. Next, to test whether

even at low level whether SHH maintains the disc health, we conditionally targeted Shh in adult mouse NP cells and found that within two months large uninucleated NP cells differentiated into small-multinucleated syncytium. Then we using tet-on system we conditionally and transiently overexpressed recombinant Shh (rShh) in the adult mouse NP cells and allowed the mice to age for another six months. While the NP cells of the control mice underwent pathological changes to form a multinucleated syncytium, those of the rShh mice maintained their large uninucleated structure. Finally, using a SmoM2 allele, we conditionally activated hedgehog signaling in the NP cells of aged mice when the NP cells normally changed from large uninucleated cells into small-multinucleated syncytium and analyzed the discs one month later. Interestingly, all NP cells were large and uninucleated again. The change in NP cell phenotype in all the genetic experiments was associated with regulation of downstream targets of SHH, including patched1, Gli1, and extracellular matrix proteins. These findings show that SHH regulates cellular plasticity of NP cells, and has the potential to rejuvenate degenerating discs.

P690/B707

Alteration of Fatty Acids Oxidation by Increased Cpt1a on Senescent of Placenta-derived Mesenchymal Stem Cells by Long-term Cultivation.

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Human placenta-derived mesenchymal stem cells (PD-MSCs) have potentials including self-renewal activity as well as differentiation into several lineages for powerful sources for cell therapy in regenerative medicine. Nevertheless, PD-MSCs shows limited lifespan according to long-term cultivation and are well unknown on cellular mechanism related to mitochondrial function. Therefore, the objectives in the study are to compare differences including alteration in cellular metabolism as well as mitochondrial function between early passage (<passage.8, Early) and late passage (<passage.14, Late) of PD-MSCs and investigate whether the expression of CPT1A is associated with mitochondrial dysfunction observed in senescent of PD-MSCs by long-term cultivation. Also, immortalized PD-MSCs (by overexpressed hTERT gene transfection) after passage number 14 were positive control of non-senescent cells. We analyzed the characterization of PD-MSCs for stemness, differentiation, cell death (e.g., apoptosis and autophagy) and mitochondrial function by qRT-PCR, western blot, Elisa, immunofluorescence and XF-assay compared with “Early” PD-MSCs. The “Late” PD-MSCs decreased the expression of stemness markers including differentiation and proliferation, otherwise the expression of senescent markers such as SA-β-gal and p21 including cell death increased compare with “Early” PD-MSCs (*p<0.05). Also, ROS, ATP production, mitochondrial membrane potential (MMP) and cellular metabolism, which are involved in mitochondrial function, were decreased in “Late” PD-MSCs compared to those of “Early” PD-MSCs (*p<0.05). Interestingly, “Late” PD-MSCs increased the expression of CPT1A, p-ACC and PPARα, which involved in mitochondrial metabolism of fatty acid, compared to those of “Early” PD-MSCs (*p<0.05). Down-regulated CPT1A by inhibitor in “Late” PD-MSCs increased MMP and decreased mitochondrial mass, ROS and ATP production compared to control (*p<0.05). Moreover, inhibited of CPT1A in PD-MSCs were increased and glucose and fatty acid mechanism were decreased compared with control (*p<0.05). These findings mean that CPT1A play as an important factor in mitochondrial function via regulation of energy metabolism and ROS level in replicative senescence of PD-MSCs according to long-term cultivation. Therefore, these data help understanding the fundamental mechanism of self-renewal of MSCs and support to overcome the replicative senescence of MSCs. This

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P691/B708

Metformin Alleviates Altered AMPK Activity Associated with a Missense Mutation in Ctrp5 Underlying Late-onset Retinal Degeneration.

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Late-onset retinal degeneration (L-ORD) is a rare disease that shares clinical phenotypes with age-related macular degeneration (AMD). Previous reports have linked compromised functionality of the retinal pigment epithelium (RPE), a critical retina layer, to the initiating events of retinal degeneration. The RPE maintains the health and integrity of the lipid and carbohydrate rich photoreceptors, and its degeneration is likely the cause of altered lipid metabolism observed in L-ORD and AMD disease pathologies. L-ORD stems from a missense mutation in CTRP5, which is highly expressed in the RPE. Using induced pluripotent stem cell (iPSC) technology, we differentiated iPSC-RPE (iRPE) cells from fibroblasts of L-ORD patients and their unaffected siblings. Mutant CTRP5, in L-ORD patient iRPE, had reduced binding affinity for adiponectin receptor 1 (AdipoR1), which led to elevated levels of pAMPK under baseline conditions (cells fed with 5% fetal bovine serum containing media). However, under conditions of further metabolic stress (ex. serum starvation or alterations to the ratio of ATP to AMP) L-ORD patient iRPE did not exhibit any additional increases in pAMPK levels. This metabolic dysfunction caused L-ORD patient iRPE to dedifferentiate and enter the process of epithelial to mesenchymal transition. Administration of metformin, an anti-diabetic drug, to L-ORD patient iRPE rescued the cells from EMT, resensitized AMPK activity, and improved lipid metabolism. Furthermore, a retrospective clinical study showed that patients ages 50-59, who had been prescribed Metformin, experienced a delayed age of onset for exudative AMD by two years. With this, we believe that metformin could be a promising course of treatment to mitigate some cellular metabolic stress that emerges during the initiating events of AMD-like retinal diseases.

P692/B709

Dysregulation of Nsun2 Promotes Pathological Tau Alterations in Alzheimer's Disease.

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Background: Currently, the molecular basis of Alzheimer's disease (AD) are unclear. However, several studies suggest that altered microRNA (miRNA) expression and/or function plays an important role in the pathogenesis of AD by altering tau proteostasis. MiRNAs are a part of vital regulatory mechanism that prevents the deposition of tau protein. However, mechanisms governing how miRNAs are regulated in the brain are poorly understood. Methylation is a prevalent posttranscriptional modification found in coding and non-coding RNAs that regulates accuracy of translation initiation, stability and biogenesis or processing. Recently, miRNAs have also been found to be targets of RNA methylation. One of the

methyltransferases specifically enriched in the brain is NSUN2. Importantly, deficits in memory and learning have been observed in NSUN2-deficient *Drosophila melanogaster* model and transgenic mice NSUN2 knockout model, indicating a potential role of NSUN2 in cognitive function. Notably, in humans, mutations in the NSUN2 gene can cause disorders that are associated with intellectual disability. Therefore, our objective is to investigate the role of RNA methyltransferase, NSUN2 in tau proteostasis regulation. **Methods:** Here we performed histological and biochemical analyses of post-mortem human brains from AD patients and healthy controls. In order to mimic pathological conditions of AD brain *in-vitro*, we treated primary neuronal cultures with synthetic oligomeric Amyloid beta (Aβ) and analyzed the effects of alterations in the levels of NSUN2 on tau proteostasis using a myriad of immunocytochemical and biochemical approaches. In addition, we chose *Drosophila* as our *in-vivo* model system to study NSUN2-microRNA modulation of tau neurotoxicity and behavior. **Results:** Our data supports dysregulation of NSUN2 in post-mortem brain tissue from AD patients when compared to healthy controls. In addition, we found that oligomeric Aβ induces both dysregulation of NSUN2 and changes in tau proteostasis in primary neuronal cultures. Furthermore, bioinformatic analysis shows predicted methylation sites in miRNAs that have been implicated in AD. Strikingly, our *in-vivo* data shows that overexpression of NSUN2 can rescue tau induced toxicity. **Conclusion:** We propose that NSUN2 mediates methylation of brain miRNAs promoting alterations in tau proteostasis leading to neurodegeneration and cognitive dysfunction. Discovering the earliest events driving alterations in tau proteostasis will identify possible therapeutic targets to slow and/or halt the progression of the disease.

P693/B710

Vascular and Neurogenic Rejuvenation in Aging Mice by Modulation of Asm.

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Although many reports have revealed dysfunction of endothelial cells in aging, resulting in blood-brain barrier (BBB) breakdown, the underlying mechanism or mechanisms remain to be explored. Here, we find that acid sphingomyelinase (ASM) is a critical factor for regulating brain endothelial barrier integrity. ASM is increased in brain endothelium and/or plasma of aged humans and aged mice, leading to BBB disruption by increasing caveolae-mediated transcytosis. Genetic inhibition and endothelial-specific knockdown of ASM in mice ameliorated BBB breakdown and neurocognitive impairment during aging. Using primary mouse brain endothelial cells, we found that ASM regulated the caveolae-cytoskeleton interaction through protein phosphatase 1-mediated ezrin/radixin/moesin (ERM) dephosphorylation and apoptosis. Moreover, mice with conditional ASM overexpression in brain endothelium accelerated significant BBB impairment and neurodegenerative change. Overall, these results reveal a novel role for ASM in the control of neurovascular function in aging, suggesting that ASM may represent a new therapeutic target for anti-aging.

P694/B711

Assessing the Genetics of Aging in the *Drosophila* Midgut.

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Aging is characterized by the progressive decline in physiological processes, including gene misregulation and loss of tissue structure and function. The mechanisms that drive aging are poorly understood. The *Drosophila* posterior midgut has emerged as an excellent model for aging given its

highly stereotypical yet simple architecture. Similar to the mammalian intestinal epithelium, the *Drosophila* midgut epithelium is controlled by intestinal stem cell proliferation and the differentiation of their progeny. The midgut contains only four basic cell types: Intestinal stem cells give rise to either enteroendocrine cells or enteroblasts which then differentiate into enterocytes (large absorptive cells). As *Drosophila* age, there is a marked decrease in the levels of adherens junctions between enterocytes as well as accumulation of undifferentiated progenitor cells. To understand how this phenotype arises, it is important to understand the gene expression changes that are occurring with age. RNA-Seq of young and old flies has unveiled a number of novel genes whose expression demonstrates the same direction of age-dependent changes regardless sex or rearing condition (up or down). To understand how candidate genes influence gut health during aging, we use the GAL4/UAS system to perform RNAi and overexpression screens. To monitor the effect of gene expression on gut health, we assessed both gut morphology and the expression profiles of other age-regulated genes. For the former, we analyzed the integrity of cell-cell contacts and accumulation of stem/progenitor cells using immunofluorescence (IF) analysis of midguts from young (7d) and old (40d) flies. For the latter, we performed RNA-Seq analysis on midguts at the same timepoints. Through these screens, we have identified genes whose knockdown improved gut health outcomes as monitored both by IF and RNA-Seq. We are currently working to pinpoint the cell type(s) in which expression of these genes is most crucial. Our future studies will analyze the mechanisms through which these candidates influence gut aging and overall organism health.

P695/B712

***Ecto*-5´-nucleotidase (CD73) Supports AMPK Activation in Hepatocytes during Physiological Liver Aging.**

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The enzymatic dephosphorylation of 5´-nucleotides, such as adenosine 5´-monophosphate (AMP) is a key step in purine salvage and purinergic signaling. This reaction occurs inside the cell or in the extracellular space. *Ecto*-5´-nucleotidase (CD73) is the major enzyme catalyzing the formation of extracellular adenosine from AMP. CD73-generated adenosine controls tissue homeostasis and responses related to inflammation, ischemia, fibrosis, and cancer. Adenosine is particularly important for metabolic homeostasis in the liver, but the specific functions of CD73 in liver homeostasis have not been elucidated. The objective of the present study was to establish a liver-specific CD73 knockout model and use it to evaluate the in *vivo* roles of CD73 in hepatocytes. We generated a hepatocyte-specific CD73 knockout mouse model (cKO) on the C57/BL6 background using the Cre-lox system. Male and female WT and cKO mice and liver tissues were compared by serological, histological, and biochemical methods. Primary hepatocytes were isolated from the mice and grown in 3D culture for molecular and imaging analyses. Loss of hepatocyte CD73 resulted in 70-80% reduction in total liver CD73 protein ($p<0.0001$). CD73 loss was associated with a significant decrease ($p<0.01$) in the activity of AMP-activated protein kinase (AMPK), assessed by substrate profiling. *Ex vivo* studies in primary hepatocytes showed that AMPK activity is dependent on extracellular adenosine, generated by CD73 and taken up intracellularly by the equilibrative nucleoside transporter ENT1. Male and female cKO mice developed normally for up to 21 weeks of age without displaying significant liver phenotypes. However, between 21-41 weeks of age, the cKO mice developed spontaneous liver injury with elevations in serum liver injury markers ($p<0.05$ in males; $p<0.15$ in females). A significant increase in lobular inflammation

and hepatocyte ballooning degeneration were observed in male cKO mice compared to male WT mice ($p<0.05$). Although AMPK hypo-activation was also observed in the livers from female cKO mice, they did not display a statistically significant difference in liver injury compared to female WT mice. A compensatory induction in adenosine receptor expression that was present in female, but not male, cKO mice may partially explain the sex differences in the development of spontaneous liver injury. In conclusion, we developed a novel mouse model to address the importance of extracellular adenosine production by hepatocytes. Furthermore, we showed that this pathways is critical for sustaining AMPK activity in the aging liver via an outside-in signaling mechanism mediated by CD73.

P696/B713

Widespread Accumulation and Aggregation of IF Proteins during Mammalian Aging and Partial Reversal by Lifelong Caloric Restriction.

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Background and hypothesis: Intermediate filament (IF) proteins are critical regulators of cellular function and are prone to misfolding and aggregation under chronic stress conditions. Many late-onset diseases exhibit perturbed proteostasis, which is one hallmark of cellular stress. Caloric restriction (CR) increases stress resistance and slows aging in multiple organisms, including non-human primates. We hypothesized that mammalian IF proteins accumulate at an organism-wide level in response to aging stress. **Methods:** We compared tissue-specific IF gene and protein expression in young (3 months old) and old (24 months old) mice from three genetic backgrounds (CBA, BALB/c and CB6F1). Brain, heart, lung, liver, intestine, pancreas, kidney and spleen tissue from male and female mice (n=3 for each sex/strain) were used for RNA and protein extraction. Tissues and serum were additionally collected from 12- and 24-month old male C57BL/6J mice that were raised on an unrestricted (ad lib; AL) or a 30% CR diet (n=3/group). Study animals were provided by the National Institute of Aging and used under an approved protocol. We used sequence-specific primers and qPCR to measure mRNA levels of *Gfap, Nefl, Nefm, Nefh, and Nes* in brain; *Des* in heart; *Vim* in heart and spleen; and *Krt8* in lung, liver, intestine, pancreas and kidney. Parallel immunoblotting on total tissue lysates compared monomeric and high molecular mass (HMM) IF protein aggregates under reducing and non-reducing conditions. **Results:** All IF proteins showed accumulation in aged animals at the level of the monomer, HMM aggregates, or both. The most prominent were changes in the heart (desmin) and lung tissue (K8). Desmin monomer (50 kDa) and HMM oligomers (>250 kDa) increased 11-fold and 7-fold, respectively in heart lysates from old versus young mice independent of sex and strain ($p<0.001$). CR diet mice had significant reduction ($p<0.05$) in serum glucose compared to AL mice (300+36 vs. 181+9 mg/dL; non-fasting state) and had 5-fold and 2-fold decrease in HMM desmin at 12 and 24 months of age, respectively ($p<0.01$). Old mice exhibited >7-fold in HMM K8 (>250 kDa) in lung tissues irrespective of sex and strain ($p<0.01$), while CR mice had 2.5-fold decrease in K8 aggregates in the lungs at 12 and 24 months (n=3/group, $p<0.01$). In general, mRNA levels did not follow protein expression changes during aging, suggesting post-translational mechanisms. **Conclusions:** We reveal widespread accumulation of IF proteins in aged mice regardless of sex or strain, and partial reversal by caloric restriction and improved glucose homeostasis.

P697/B714

Assessing the Role of the Progeria Associated BAF A12T Variant on Nuclear Envelope Integrity, Chromatin Conformation and Genotoxic Stress.

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Among the defined hallmarks of cellular aging are DNA damage, genomic instability, epigenetic alterations, cellular senescence, oxidative stress and stem cell exhaustion. Premature aging syndromes (progerias) are useful models to complement the study of physiologic aging. A subset of premature aging syndromes arises from mutations in the components of the nuclear envelope resulting in what is known as a nuclear envelopathy. The most common and best studied of these progerias, Hutchinson Gilford Progeria Syndrome (HGPS), arises from an autosomal dominant lamin A/C mutation that leads to the accumulation of a variant of lamin a and a characteristic nuclear envelopathy. More recently, Nestor Guillermo Progeria Syndrome (NGPS) has been identified, resulting from the homozygous inheritance of a BANF1 mutation causing an Ala-Thr substitution at residue 12 of the BAF protein ($A^{12}T$). BAF is a small, essential, dimeric dsDNA-binding protein that serves as a bridge between interphase chromatin and the nuclear envelope. BAF has been implicated in regulating chromatin conformation and the DNA damage response and also participates in the recognition and repair of nuclear envelope ruptures and the enclosure of mitotic chromosomes in a single nuclear envelope. NGPS patient-derived fibroblasts exhibit nuclear envelopathy, and patients exhibit signs of premature aging, including progressive osteolysis and loss of subcutaneous fat. Our interest lies in the overlap between disruptions in the nuclear envelope, chromatin conformation and genotoxic stress. To address the cellular and organismal pathology of NGPS, we have used CRISPR/Cas9 technology to establish BAF^{A12T}/BAF^{A12T}human induced pluripotent stem cells (hiPSCs). The BAF^{A12T}iPSCs appear normal, but in comparison to isogenic control cells, their ability to differentiate into mesenchymal stem cells (MSCs) in vitro is incomplete. BAF^{A12T}MSCs exhibit a marked increase in cellular senescence and an elevated cellular stress response. We are addressing the underlying cause of the senescent phenotype as well as monitoring BAF^{A12T}-induced changes in chromatin conformation, DNA damage and oxidative stress. We have also used CRISPR/Cas9 technology to establish a colony of BAF^{A12T}/+ mice. Mice that are homozygous for the $BAF^{A12T/A12T}$allele are viable and primary cultures of fibroblasts derived from the ears of mice are not senescent and do not demonstrate elevated basal DNA damage; however, these cells do show a marked nuclear envelopathy. Further characterization of these fibroblasts as well as mouse-derived MSCs are planned. Taken together, these in vitro and in vivo studies are aimed at uncovering the cellular and organismal impact of the BAF ^{A12T}mutation.

P698/B715

Effects of Age-dependent Changes in Cell Size on Endothelial Cell Growth through Yap1.

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Introduction:The aging population is rapidly growing. Aging is associated with impaired angiogenesis - the growth of new blood capillaries- and contributes to the increased susceptibility to various diseases. Thus, in order to develop more efficient therapies for aging-associated diseases, we need to understand the mechanisms by which aging impairs angiogenesis. In addition to soluble angiogenic factors, biophysical factors such as changes in cell size and geometry control endothelial cell (EC) growth and differentiation. A Hippo signaling transducer, Yes-associated protein (YAP1), acts as a mechanosensitive transcriptional co-activator and controls angiogenesis and organ regeneration. Methods: We use silver

nitrate staining and immunocytochemical analysis to measure the EC size in blood vessels ex vivo and in vitro. To directly examine the effects of cell size on YAP1 activity and EC proliferation and senescence, we use the microcontact printing system and culture young vs. Aged ECs on each island. Results: We have found that pulmonary artery ECs in aged mice are significantly larger than those in young mice. Aged human adipose tissue ECs are also larger than those in young adults. A major focal adhesion protein, paxillin, distributes at the distal ends of actin stress fibers in young ECs, while paxillin is localized along the actin fibers in the cytoplasm in aged ECs. The levels of YAP1 decrease and EC senescence is induced in aged ECs. The activity of CDC42, which controls actin cytoskeleton structures, is also higher in aged ECs compared to that in young ECs. To analyze whether age-related changes in EC size control YAP1 activity and EC senescence, we culture ECs on the microcontact-printed substrates consisting of square fibronectin-coated single-cell sized islands. When we culture aged human adipose ECs on large islands of size comparable to aged EC, YAP1 is excluded from the nucleus and EC proliferation is attenuated. Reduction of the aged EC size by culturing on smaller islands restores YAP1 nuclear localization, decreases Cdc42 activity, and inhibits EC senescence. Stimulation of YAP1 or inhibition of Cdc42 activity in aged ECs also restores blood vessel formation. These results suggest that age-dependent increases in EC size stimulate aged EC senescence through CDC42-YAP1 signaling. Conclusion: Modulation of EC size and/or Cdc42 and YAP1 activity will reverse age-related decline in angiogenesis and will lead to the development of promising strategies for age-dependent diseases.

P699/B716

A Role for Unc-45 in Maintaining Myosin during Aging.

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UNC-45 is a chaperone required for the folding of functional myosin heads and the proper assembly of myosin into thick filaments. UNC-45 was first discovered in *C. elegans* and later found to be conserved in all animals. UNC-45 consists of a TPR domain that interacts with HSP-90, a central domain, and a UCS domain that interacts with and folds the myosin head. In addition to its essential role in muscle development, we hypothesize that UNC-45 has a role in mature muscle, to re-fold myosin heads damaged from thermal or oxidative stress. One type of stress is aging, as a popular theory is that the decline in cellular function found in aging is due to an accumulation of damage to macromolecules that occurs with time. Sarcopenia is the decreased muscle mass and function seen in the elderly in the absence of any underlying disease. Herndon et al. (2002) showed that *C. elegans* can serve as a model for sarcopenia. Using immunostaining with antibodies to myosin heavy chain a (MHC A), we show that there is a gradual decline in the number of A-bands (a measure of thick filament assembly) beginning at day 8 adults. By day 12 and especially day 16, there is also disorganization of A-bands. This disorganization appears similar to that of *unc-45* ts mutants grown at the restrictive temperature. By quantitative western blotting, we examined the levels of UNC-45, HSP-90, MHC a and MHC B, and found that all decline with age. Whereas MHC a and HSP-90 show a gradual decline, UNC-45 shows a major decline between days 3 and 4, and MHC B shows a major decline between days 4 and 8. Since the steady state *unc-45* transcript level shows either no change or an increase during aging, this suggests an increase in UNC-45 degradation over time. Unlike *unc-45*, *hsp-90* transcript levels decrease along with the protein. We also observe a decrease in UNC-45 protein, but not mRNA, in an *hsp-90* loss of function mutant, suggesting a role for HSP-90 in UNC-45 regulation and/or protein stabilization. Preliminary results indicate that UNC-45 protein and transcript increase with heat stress, providing further support for the role of UNC-45 in re-folding myosin heads after stress. We are testing the hypothesis that at least

some of the decrease in myosin heavy chain levels and thick filament assembly that occurs with aging is due to a decline in the level of UNC-45.

P700/B717

Using Zebrafish to Correlate Hair-cell Presynaptic Activity with Ototoxin Resistance.

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Hair cells, the primary sensory cell of the auditory system, rely on calcium for their development and function. Exposing these cells to known ototoxins such as aminoglycoside antibiotics can cause irreversible hearing loss. Differential susceptibility to aminoglycosides has been reported in hair cells of various species including those of the zebrafish lateral line, but the mechanism behind this phenomenon is unknown. Functionally, sensory stimuli activate hair cells by eliciting the opening of basal presynaptic calcium channels followed by glutamate release onto afferent neurons. We have discovered that this synaptic activity is only detectable in approximately 30% of zebrafish hair cells and classified these as synaptically “active”. Because of the importance of calcium homeostasis in hair-cell survival, we hypothesized that differential susceptibility to aminoglycoside ototoxicity in zebrafish hair cells may be related to the presence or absence of presynaptic calcium signals. To explore this further, we are conducting functional calcium imaging in transgenic zebrafish expressing genetically encoded calcium indicators (GCaMPs) in external lateral-line hair cells. GCaMP imaging enables us to measure evoked presynaptic calcium signals within hair cells in *vivo*. After collecting activity measurements, we apply the aminoglycoside neomycin or neomycin-Texas Red solution to larvae, track cell survival, and draw correlations between survival, activity, and drug uptake. We are complementing these analyses by employing genetic mutants or pharmacological agents that chronically or transiently block presynaptic calcium signaling, respectively. Currently, we have found that synaptically “active” cells are more resistant to aminoglycoside ototoxicity. Additionally, the majority of “active” cells contain significantly less neomycin than “inactive” cells. Although the exact mechanism behind this is unclear, our preliminary data suggests that “active” cells have a younger cumulative age, rendering them more resilient to aminoglycosides. We have examined the relationship between presynaptic calcium signaling and aminoglycoside ototoxicity more closely following the chronic or acute disruption of presynaptic calcium. We found that chronic but not acute disruption of this activity actually augments cell survival. We are currently expanding our analyses to investigate the relationship between presynaptic activity, cumulative cell age, and cell death mechanisms. Overall, understanding how activity-dependent signals provide protection is critical to the development of otoprotective or even regenerative therapies.

P701/B718

Identification of Novel Interacting Proteins of Small Heat Shock Rotein Hspb5 by Using Bioid2 Method.

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HSPB5 belongs to a small heat shock protein (HSPB) family and highly expressed in skeletal muscle. HSPB5 is regulated by the phosphorylation with various cellular stresses and exhibits a cytoprotective function. Since HSPB5 expression is markedly increased in skeletal muscle of aging mice, it is speculated that HSPB5 plays a role in the endoplasmic reticulum (ER) stress by senescence. To elucidate the

resistance of HSPB5 to cell senescence, we searched for the binding protein of HSPB5 by using biotin enzyme labeling with BioID2. HSPB5-BioID2 biotinylated a protein near HSPB5 under ER stress conditions with the proteasome inhibitor MG132. After digestion of the cell lysate with trypsin, the biotinylated peptides were purified with tamavidin 2-REV beads and subjected to nanoLC-MS/MS. As a result, 91 biotinylated proteins were identified. In this study, we examined polo-like kinase 2 (PLK2), an apoptosis-related enzyme, as a candidate for a new binding protein. After MG132 treatment, HSPB5 and PLK2 protein expression levels were increased and co-localization was observed in the ER in myocytes. The binding of HSPB5 and PLK2 by MG132 treatment was confirmed by immunoprecipitation. Moreover, PLK2 inhibitor BI2536 suppressed HSPB5 phosphorylation and translocation of HSPB5 to ER by MG132. These results suggest that HSPB5 translocates to the ER through the phosphorylation by PLK2 and plays some cytoprotective function against ER stress.

P702/B719

Cellular Mechanisms of Aging within the Stem Cell Niche.

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Stem cells self-renew, and can also produce daughter cells that will differentiate into the specialized cell types that are important for function and repair of their resident tissue. Many tissues become compromised as they age, raising major questions about how stem cell behavior is affected during aging. Aging might affect factors intrinsic to stem cells, or extrinsic factors from their environment, since niches often influence decisions between self-renewal and differentiation. The *Drosophila melanogaster* testis is a well-studied tissue that has served as a paradigm for our understanding of communication between the stem cells and their niche. Niche signals maintain germline stem cells (GSCs) in several ways. One particular function acts to orient GSC divisions so that differentiating daughter cells move out of the influence of the niche, and therefore can eventually produce sperm. For this division to be oriented properly, the stem cell centrosome is anchored at the interface with the niche, and this relies on factors such as E-cadherin and Apc2 within stem cells (Yamashita et al, 2003; Inaba et al, 2010). Previous work in our lab showed that one signal secreted by the niche, Unpaired, maintains E-Cadherin-based adhesion of the GSC to the niche (Leatherman and DiNardo, 2010). Since others have shown that this signal decreases with age, we wished to test whether aging might reduce the fidelity of the oriented divisions (Toledano et al, 2012). We quantify oriented divisions using live imaging and other tools to extract the division axis in 3D space relative to the niche/stem cell interface (Jüschke et al, 2014). Preliminary data showed that in aged testes orientation was indeed compromised, as there was less of a bias towards division away from the niche. This suggests that the fidelity of oriented divisions has been compromised with age. Assessing such changes in stem cell dynamics upon aging will help us asses how stem cell-niche communication is disrupted with age, and will thus fill an important gap in our knowledge.

P703/B720

Bax Inhibitor-1 (bi-1) Regulates D-galactose-induced Cellular Senescence.

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ER stress is one of the critical features in senescence. Bax Inhibitor-1 (BI-1) is an evolutionary-conserved endoplasmic reticulum (ER) protein that regulates cellular response to ER stress and apoptosis.

However, the role of BI-1 in regulation of senescence remains elusive. In this study, we aim to assess whether BI-1, an ER stress regulator, participates in regulating drug-induced cellular senescence. Using D-galactose and tunicamycin as an aging and ER stress inducer respectively, we demonstrated and compared different ER stress markers in hepatic cell lines. Palmitate (250 μM), a saturated fatty acid was used to induce lipid-mediated ER stress. Senescence markers such as p53 and p21 as well as ER stress markers such as GRP78, CHOP, p-IRE1a, sXBP1, and p-eIF2a were analyzed by western blotting using various concentrations of D-galactose and palmitate. D-galactose treatment in Hep3B cells induces expression of p53, p21 and CHOP expression. Interestingly, combined treatment of D-gal and palmitate induced senescence as well as the expression of ER stress proteins. These experiments were conducted and compared in HA-tagged BI-1 overexpressed cells and with neomycin resistance pcDNA3 hepatic cells. We present evidence that senescence and ER stress-induced ROS was decreased in BI-1 overexpressed cells. The expression of ER stress response proteins such as GRP78, phosphorylation of IRE1α and eIF2α, spliced XBP1 and CHOP were analyzed and found that the BI-1 showed the inhibitory response of ER stress in D-gal, palmitate, tunicamycin or combined treated HepG2 and Hep3B cells. Moreover, D-gal, palmitate, tunicamycin, combined treatment of D-gal and palmitate, or with tunicamycin induced cleavage of caspase-7 and caspase 12 were also alleviated in BI-1 overexpressed cells, regulating ER stress-mediated apoptosis. Together, our findings revealed that BI-1 regulates and protects hepatic cells from senescence-mediated ER stress and suggests that activation of BI-1 has the potential to alleviate D-gal-induced senescence. **Keywords:** Bax Inhibitor-1, D-galactose, senescence, ER stress, apoptosis

P704/B721

Temporal Control of FOXO Expression Levels Alleviates Acute High Dose-induced Cardiac Dysfunction in Young and Aged *Drosophila*.

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Cardiovascular disease (CVD) is one of the leading causes of death worldwide, and the risk of developing it increases with age. *Drosophila melanogaster* is a rapidly aging animal model whose limited genetic variability and cellular complexity allows for comprehensive exploration of cardiac senescence within a short time frame relative to other standard model organisms. The transcription factor FOXO has been associated with longevity and health-span for some time and regulates expression of genes involved in many cellular processes. Previously, mild cardiac-restricted dFOXO overexpression in *Drosophila* provided cardioprotection accompanied by increased expression of genes associated with the ubiquitin-proteasome system (UPS) at advanced age. Increasing the dosage of dFOXO, however, proved lethal. As a transcription factor, FOXO must be activated in specific amounts for proper cellular function, but this activity is likely weakened over time, contributing to cellular senescence. This has not yet been shown explicitly in animal models or aged human samples. We hypothesized that overexpressing FOXO later in life would alleviate the negative effects observed in young flies and potentially improve heart function at advanced age. To test this hypothesis, we have used a GeneSwitch heart-specific GAL4 driver. Overexpressing moderate doses of dFOXO using mifepristone, RU486, beginning at week 3 resulted in unimpeded heart function. This is in contrast to previous results showing that even mild overexpression of dFOXO using RU486 at 2-days post-eclosion caused heart function decline in young flies. Furthermore, when these flies were aged to 6 weeks, elderly for a fruit fly, they experienced ameliorated cardiac decline compared to flies given vehicle.

P705/B722

Progerin Expression Causes Splicing Mis-regulation in Non-hgps Patients.

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Hutchinson-Gilford progeria syndrome (HGPS) is a rare, autosomal dominant disorder caused by a mutation in the *LMNA* gene that activates a cryptic splice site whose use results in a mutant lamin a protein product known as progerin. Progerin interferes with lamin a protein function in a dominant negative manner and causes nuclear abnormalities, genomic instability, and altered redox homeostasis. Remarkably, progerin is also found in normally aging individuals. Progerin production appears to be induced by various types of DNA damage, including DNA double-strand breaks (DSBs) induced by telomere shortening or UV light. Furthermore, the preliminary analysis in hundreds of tissue samples from the Genotype-Tissue Expression Project (GTEx) reveals striking correlations between progerin expression and the expression of genes known to regulate RNA splicing. My studies seek to elucidate the causal relationships between DNA damage, altered splicing, progerin production, and downstream effects.

P706/B723

The Recovery Effect of Mitochondrial-derived Peptide Mots-c to Aged Human Placenta-derived Mesenchymal Stem Cells.

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The aging of the mesenchymal stem cell (MSC) is one of the major reasons behind the cause of diseases such as the regeneration disorder, and it is related to metabolic homeostasis in the cells. However, recovery factors of aged MSC are not completely elucidated for the MSC. Loss of cellular function in aging is associated with metabolic homeostasis and oxidative stress such as the ROS production. Recently, the mitochondrial open reading frame of the 12S rRNA-c(MOTS-c) was reported to have played a role for the metabolic homeostasis activity. But specific effects of MOTS-c were not investigated for the recovery of aged the MSC for humans. Here we investigate the recovery of aged MSC with MOTS-c through metabolic homeostatic. We prepared and maintained human chorionic plate-derived mesenchymal stem cells (hCP-MSC) donated by our colleague. An d we made old passage hCP-MSC as aged cells by in *vitro* overpassing subculture from young hCP-MSC. Then we confirmed phenotypes of the aged hCP-MSC such as cellular morphology, doubling times and several senescence associated genes and proteins expression like AMPK, SIRTs, FOXO, HO-1/2 and SOD1/2. The activated form of AMPK by phosphorylation was also confirmed as AMPK is a major target of MOTS-c. As a result, aged MSCs exhibit a larger cell size compared to young MSCs. But MOTS-c treated aged MSCs decrease in cell size compared to aged MSCs. In addition, MOTS-c treated aged MSCs showed to appear as a more spindle-like shape like young hCP-MSC while aged MSCs showed a flatter and an abnormal cellular shape. Above these, aged hCP-MSC showed to significantly decrease in doubling-time and lipid droplet formation by MOTS-c. Therefore, aged MSCs that were treated with MOTS-c have significantly increased gene and protein expression level in senescence associated genes compared to the aged MSCs. It's mean cellular homeostasis was recovered as a young hCP-MSC from aged hCP-MSCs. Phosphorylated-AMPK was also evaluated and showed similar positive results as well. In conclusion, we found out that the rejuvenation effects of MOTS-c for aged MSCs was similar to the way that the genes associated with

cellular homeostasis and prevention of ROS damage act. Also, recovered cellular morphology and decreased doubling-time and lipid droplet formation also supported the rejuvenation effects of MOTS-c. Therefore, after further study, we want to apply to in vivo study for clinical application.

P707/B724

A Novel Regulator of Branched-chain Amino Acid Homeostasis Controls the Actin Cytoskeleton and Extends Lifespan in *Saccharomyces Cerevisiae.*

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Previous studies in our laboratory revealed that the membrane cytoskeletal interactions that support mitochondrial inheritance results in preferential inheritance of fitter mitochondria by yeast daughter cells, which promotes daughter cell fitness and lifespan. Specifically, we found that actin cables, bundles of actin filaments that align along the mother-bud axis and serve as tracks for movement of mitochondria from mother to daughter cells, are dynamic structures that move in the retrograde direction, from bud to mother cells. As a result, mitochondria are effectively “swimming upstream” against the opposing force of retrograde actin cable flow (RACF) as they move from mother to bud cells, thus, the bud cells’ preferentially inherit higher functioning mitochondria that are more motile and have a higher membrane potential. This, in turn, contributes to mother-daughter age asymmetry, the process whereby mother cells continue to age as they give rise to daughter cells that are, for the most part, born young and with a full replicative lifespan. Here, we report that the stabilization of actin cables can also promote mitochondrial fitness and extend replicative lifespan, and a role for a previously uncharacterized open reading frame (ORF) in that process. Using a genome-wide screen, we found that the deletion of an ORF reduced the sensitivity of yeast to growth inhibition induced by the actin destabilizing drug, Latrunculin-A (Lat-A). Yeast bearing a deletion in this gene also displayed an increase in actin cable abundance and stability, increased mitochondrial fitness, and extended lifespan. Transcriptome analysis revealed that deletion of this ORF results in decreased branched-chain amino acid (BCAA) biosynthesis and increased BCAA degradation. Consistent with this, we find the deletion of BCAA transaminase produces cells with increased actin cable abundance and mitochondria fitness, which are similar to those observed upon deleting the ORF. Furthermore, physically changing external BCAA levels also result in the same morphological changes in the actin cytoskeleton. Moreover, we find that the observed phenotypic changes occur in a TORC1-independent manner. Finally, we find that stabilizing actin promotes mitochondrial function and the fitness of yeast as they age. Our studies revealed a novel regulator of BCAA metabolism, and a role for BCAAs in the actin cytoskeleton regulation, which in turn, increases mitochondria quality and extends longevity. An alysis of this newly identified factor will not only expand our understanding of the interconnection between nutrients, the actin cytoskeleton and aging, but also provide a foundation for developing aging interventions.

Ubiquitin and Proteasome Function

P708/B725

Molecularly Distinct Cores Coexist Inside Stress Granules.

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Stress granules are membraneless organelles that form in eukaryotic cells after stress exposure. Stress granules are constituted by a stable core and a dynamic shell that establishes a liquid-liquid phase separation with the surrounding cytosol. The structure and assembly of stress granules and how different components contribute to their formation are not fully understood. Here, using super resolution and expansion microscopy, we find that the stress granule component UBAP2L and the core protein G3BP1 occupy different domains inside stress granules. Since UBAP2L displays typical properties of a core protein, our results indicate that different cores coexist inside the same granule. Consistent with a role as a core protein, UBAP2L is required for stress granule assembly in several stress conditions and reverse genetics show that it acts upstream of G3BP1. We propose a model in which UBAP2L is an essential stress granule nucleator that facilitates G3BP1 core formation and stress granule assembly and growth.

P709/B726

Regulation of the Unfolded Protein Response during Er Stress.

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The endoplasmic reticulum (ER) coordinates critical cellular functions such as protein folding, Ca^{2+} storage and lipid and carbohydrate metabolism. Intrinsic and extrinsic processes can disturb protein folding homeostasis. The unfolded protein response (UPR) is the principal stress signaling pathway of the ER which is activated when the ER homeostasis is disturbed. The UPR is transduced via three sensors: pancreatic ER eukaryotic translation initiation factor (eiF)-2 α kinase (PERK), inositol-requiring enzyme 1 (IRE-1), and activating transcription factor 6 (ATF6). While studying the functions of cullin 3 (CUL3) we accidentally uncovered a new link between CUL3 and the UPR. Here, we propose CUL3 as a new regulator of the UPR sensors. CUL3 is the scaffold component of CUL3-RING ubiquitin ligases (CRL3s) where BTB proteins serve as substrate-specific adaptors. Knockdown of CUL3 in human skin fibroblasts resulted in increased levels of PERK and IRE1α, but not those of ATF6. Looking further downstream the pathway, increased levels of PERK led to enhanced phosphorylation of eIF2α, a downstream effector of PERK. When the ER of the fibroblasts was stressed with DTT, CUL3 knockdown resulted in an increase of spliced XBP1, a downstream effector of IRE1α. The UPR regulation, especially of the PERK branch, by CUL3 was also observed in other cell lines (IMR90, HeLa and C2C12). However, CUL3 did not regulate the levels of IRE1α in HeLa cells and C2C12 cells. These results suggest that CUL3 regulates the UPR in a cell line-specific manner. We identified that BTB proteins such as KLHL12 and KLHL41 regulate PERK levels in the fibroblasts and C2C12 myotubes, respectively. Our data indicate that the observed cell line-specific UPR regulation depends on the profile of relevant BTB proteins in a cell line. We conclude that CUL3 represents a new class of UPR regulators. Interestingly, KLHL41 mutations have been shown to cause nemaline myopathy. The mutations cause destabilization of the sarcomeric thin filaments. Diseased muscles also exhibit atrophy and regenerative defects. However, it is unclear

how the structural defects of the filaments influence survival and regeneration of muscle. As PERK has been implicated in muscle survival and regeneration, we propose that KLHL41 mutations disturb the UPR and this UPR dysregulation contributes to muscle atrophy and regenerative defects.

P710/B727

The Effects of *Ubc-6* Mutations on Glr-1 Abundance in *C. Elegans*.

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In order for proteins to function properly, they must fold into their correct structures. Some proteins may not be able to fold correctly and can adversely affect the health of an organism, as the accumulation of misfolded proteins has been associated with several neurodegenerative diseases. To prevent misfolded proteins from aggregating, a biochemical pathway called Endoplasmic Reticulum-Associated Degradation (ERAD) ensures that misfolded proteins are degraded. ERAD utilizes several different proteins that work together to attach the protein ubiquitin to the surfaces of misfolded proteins. UBC-6 is an E2 ubiquitin conjugating enzyme that helps attach ubiquitin to misfolded proteins and is encoded by the gene *ubc-6*. GLR-1 is an AMPA-type glutamate receptor known to be involved in learning and memory. To investigate the role of ERAD components and whether or not different mutations affect GLR-1 abundance, we performed immunoblotting analysis of animals expressing GLR-1 fused to GFP (GLR-1::GFP) in a variety of different mutant backgrounds. We quantified GLR-1 protein abundance in three *C. elegans* strains with different mutations in the *ubc-6* gene. The mutations included a single point mutation, a large knock-in/substitution, and a complete excision of the coding region. Our results show that there is no significant difference in GLR-1 abundance between wild type animals and those harboring any of the *ubc-6* mutations. This suggests GLR-1 abundance isn't regulated solely by *ubc-6* and there may be other compensatory mechanisms involved in its regulation. We are currently testing the effect of loss of function mutations in another E2 gene, *ubc-7* on GLR-1::GFP abundance. This research will help us determine the importance of ERAD in regulating GLR-1. Researching the ERAD pathway in *C. elegans* is exciting not only because most of the research on ERAD has been done in yeast, but it will also help us determine whether ERAD components function in a similar manner in yeast and worms.

P711/B728

The Enzyme Activity of Ubiquitin-specific Protease 8 Is Regulated by the Intramolecular Interaction of the USP Domain and the WW Domain.

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Ubiquitin-specific proteases (USPs) are a family of deubiquitinating enzymes. USP8, a member of USPs, deubiquitinates growth factor receptors at the endosome, enhances their recycling to the plasma membrane, and thereby plays a critical role in the control of cell proliferation. However, the regulatory mechanisms of USP8 activity are not fully understood. Here, we show a novel autoinhibitory mechanism of USP8 activity. Firstly, we measured deubiquitinating activities of wild type USP8 and the deletion mutants toward ubiquitin chains in *vitro*. A deletion of a central region (aa 645-684) increased the activity, indicating that this is an autoinhibitory region. Ubiquitin-vinyl methyl ester (Ub-VME) is a probe that can covalently bind to the USP catalytic site. The deletion of the autoinhibitory region increased the in *vitro* binding of USP8 to Ub-VME. Amino acid sequence of this autoinhibitory region is partly

homologous to the WW domains of MAGI-1 and SAV-1. Amino-acid substitution of conserved tryptophan to serine (W655S) increased the USP8 enzyme activity, indicating the importance of this residue. In general, WW domain interacts with proline-rich hydrophobic sequences. Substitutions of L954 and P955 in the USP domain to alanines (L954A;P955A) also increased the USP8 enzyme activity. It raised the possibility that the autoinhibitory region intramolecularly interacts with this hydrophobic sequence in the USP domain, thereby inhibiting the USP domain-ubiquitin interaction. To test it, we constructed a single molecule FRET probe in which YFP and CFP were fused to the autoinhibitory region and the USP domain, respectively. As expected, we observed FRET signal in lysates of HEK293T cells expressing this probe. W655S mutation decreased the FRET signal, and L954A;P955A mutation also decreased it. Taken together, these results indicate the region aa 645-684 in USP8 forms functional WW domain, intramolecularly interacts with the hydrophobic sequence containing L954 and P955 in the USP domain, and inhibits the enzymatic interaction between USP domain and ubiquitin chains. Our findings may provide a better understanding of the physiological regulation of USP8 activity and fine-tuning of growth factor signaling.

P712/B729

Investigating the Mechanism of Smurf2 Regulating DNA Damage Response.

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Smad ubiquitin regulatory factor 2 (Smurf2) was originally discovered as an E3 ubiquitin ligase regulating the Smad-dependent TGF-β signaling pathway. Later on, Smurf2 was discovered to have a tumor suppressor role by targeting ring finger protein 20 (RNF20) for polyubiquitination and proteasome degradation. As RNF20 is also an E3 ligase which catalyzes the monoubiquitination of histone H2B and causes chromatin relaxation, the accumulation of RNF20 in Smurf2-/- MEFs makes the relaxed chromatin more susceptible to DNA damage, which can result in genomic instability and lead to tumorigenesis. Conversely, RNF20-H2B pathway plays a positive role in DNA damage response, where monoubiquitinated H2B recruits repairing proteins to the damage sites and facilitate the clearance of γ-H2AX, a marker of DNA damage. Smurf2 and RNF20 were found to be colocalized at the γ-H2AX foci of double-stranded DNA breaks in the nucleus, indicating that Smurf2 may also has a role in DNA damage response. As phosphorylation cascade plays an important role in regulating DNA damage response, we seek to examine Smurf2 phosphorylation upon etoposide treatment, which can induce DNA double-stranded break. Smurf2 was found to be an ATM substrate by the in-vitro kinase assay. The degradation of RNF20 by Smurf2 was triggered by etoposide treatment and blocked by an ATM inhibitor, which indicates that the phosphorylation is important for the ubiquitination process. Further we identified the phosphorylation site and made phosphorylation-block mutant. The phosphorylation mutant affected the binding between Smurf2 and RNF20, thus decreased the ubiquitination of RNF20 by Smurf2. Further, we treated Smurf2-/- MEFs stably expressing WT or mutant Smurf2 or control vector with etoposide, and we found that more cell death was observed in WT MEFs, which indicates that the phosphorylation may increase the sensitivity of the cells to etoposide treatment. In summary, we discovered that ATM-mediated Smurf2 phosphorylation is important for the ubiquitination of RNF20 by Smurf2 and Smurf2 plays a dual role in DNA damage response through degrading RNF20, by which Smurf2 makes chromatin more compact and then resistant to DNA damage while fewer repairing proteins are recruited when DNA damage happens.

P713/B730

The AUP1 UBE2G2 Binding Region (G2BR) Plays a Central Role in Activating Endoplasmic Reticulum-associated Degradation.

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The endoplasmic reticulum (ER) is the point of entry to the secretory pathway for nearly a third of newly-synthesized proteins. For this reason, ubiquitin and proteasome-mediated ER-associated degradation (ERAD), which regulates protein levels and effects quality control, is fundamental to proteostasis. In mammalian cells there are ~35 ubiquitin-conjugating enzymes (E2s) that functionally pair, in a highly selective manner, with >500 substrate-specific RING ubiquitin ligases (E3s) to mediate ubiquitination. Despite this E2:E3 selectivity in cells, in *vitro* assays demonstrate very limited selectivity in E2:E3 pairings. In ERAD, the most prominent E2 is UBE2G2. This cytoplasmic E2 has been shown to functionally interact in cells with multiple ERAD E3s, including gp78, HRD1 (Synoviolin), and TRC8. Yet, of greater than 30 potential ERAD E3s, only gp78 is known to directly recruit UBE2G2. This occurs via its UBE2G2 binding region ($G2BR^{gp78}$). Determining how UBE2G2, among all of the cytosolic E2s, is recruited to the ER to catalyze substrate ubiquitination with ERAD E3s, other than gp78, is critical to understanding proteostasis. An cient ubiquitous protein 1 (AUP1) is an ER membrane-anchored accessory protein that is required for ERAD mediated by HRD1 and TRC8, but not gp78. Strikingly, AUP1 includes a 26 amino acid cytosolic region that binds specifically to UBE2G2. This $G2BR^{AUP1}$ bears sequence similarity to $G2BR^{gp78}$. We have determined the structure of the $G2BR^{AUP1\ in}$ complex with UBE2G2. The $G2BR^{AUP1}$ forms an alpha-helix that interacts with the 'backside' of UBE2G2 via an extended interface that includes numerous electrostatic and hydrophobic contacts. An alogous to the $G2BR^{gp78}$, this interaction site is distant from both the active-site cysteine of the E2 and its canonical RING-binding interface. In *vitro*, the $G2BR^{AUP1}$ binds UBE2G2 with low nanomolar affinity, allosterically enhances UBE2G2:RING affinity, and stimulates RING-mediated ubiquitination. Although AUP1's acyltransferase and ubiquitin-binding CUE domains have been suggested to be important for ERAD, we find that a membrane-tethered $G2BR^{AUP1}$ is necessary and sufficient for degradation of multiple ERAD substrates in cells. Furthermore, we demonstrate that, in cells, the $G2BR^{AUP1}$ selectively recruits UBE2G2 to the ER membrane to target both HRD1 and TRC8 substrates. Importantly, however, membrane recruitment of UBE2G2 is not sufficient for ERAD. The $G2BR^{AUP1}$ is also required to activate this E2. Thus AUP1, through its G2BR, serves as an essential activator for multiple ERAD E3s, likely including some that await assessment, and is a key player in proteostasis.

P714/B731

Modeling Neurodegeneration in the Endoplasmic Reticulum-associated Protein Degradation Pathway Using *C. Elegans*.

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Neurons are the cells that send and receive signals, allowing animals to perceive their environment and respond to their surroundings. Proteins are essential, complex molecules that carry out a wide variety of functions within a cell. The function of a protein is determined by its shape and how it is folded. When proteins are misfolded, they may lose their ability to function correctly, accumulate within a cell, and form protein aggregates that disrupt other processes. Therefore, the ability to degrade misfolded proteins is important for maintaining healthy cellular function. Without protein regulation, protein

aggregates in neurons may cause neurodegenerative diseases such as Parkinson's and Alzheimer's disease. Endoplasmic reticulum-associated degradation (ERAD) is a ubiquitin-dependent process that allows cells to eliminate misfolded proteins and prevent toxic protein aggregation at the Endoplasmic Reticulum (ER). Ubiquitin is a small protein that is attached to misfolded proteins to mark them for proteasomal degradation, via ERAD. UBC-6 and UBC-7 are E2 ubiquitin-conjugating enzymes that are important for ERAD in yeast, but there is much less known about they may work during ERAD in neurons. We use mutational analyses to understand how vital UBC-6 and UBC-7 are in regulating neurotransmitter receptors in the nematode worm, *C. elegans*. We used two types of mutations: point mutations and gene knockouts. We hypothesized that a gene knockout of *ubc-6* or *ubc-7* could cause an increase in protein aggregation when compared with single point mutations because a larger deletion may be more severe and has been associated with behavioral defects, while a point mutation might leave the enzyme still active. In order to track neural protein aggregation, we follow the glutamate receptor GLR-1 with a green fluorescent protein (GFP) tag (GLR-1::GFP) and utilize fluorescence microscopy and FIJI image analyzing software. We found that *ubc-7* knockout mutants had a significantly higher abundance of GLR-1::GFP in cells that express the fusion protein when compared to wild-type and the other mutations. Our findings suggest that our point mutation mutants and *ubc-6* mutants still carry out ERAD, though less efficiently than wild-type. Since the knockout mutations in UBC-7 resulted in increased protein aggregation when compared with UBC-6, this suggests that UBC-7 is more vital for ERAD than UBC-6 in regulating neurotransmitter receptors. In the future, we will be creating fluorescent reporters for UBC-6 and UBC-7 to assess their expression and localization; we will also analyze GLR-1::GFP abundance for the *ubc-6* and *ubc-7* double mutants.

P715/B732

Inflammatory Bowel Disease-associated Ubiquitin Ligase RNF183 Promotes Lysosomal Degradation of DR5 and TRAIL-induced Caspase Activation.

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Ubiquitin ligase (E3) is the most critical enzyme in the ubiquitination machinery for forming polyubiquitin chains as it participates in the regulation of substrate preference and degradation rate. It has recently been reported that RNF183 expression is upregulated in intestinal epithelial cells in inflammatory bowel disease (IBD) patients and in trinitrobenzene sulfonic acid-induced colitis mice. However, the molecular mechanisms of RNF183 in the development and pathophysiology of IBD are not fully understood. Here, we used 3.5% dextran sulfate sodium (DSS) in the drinking water for 1, 3, and 5 days to establish an acute colitis model. The levels of RNF183 expression and inflammation were evaluated by quantitative PCR in terms of changes in inflammatory marker levels and by hematoxylin-eosin staining, respectively. PCR analysis revealed that RNF183 mRNA levels were significantly upregulated in DSS-induced colitis mice at 1 and 3 days after exposure and were most highly upregulated at 5 days. Although the levels of inflammatory markers, such as IL-1& beta and TNF-& alpha, also significantly increased at 5 days after exposure, their levels were not significantly changed at 1 day after exposure. From this, we speculate that RNF183 was not upregulated as a downstream target of the inflammatory response. Furthermore, we identified death receptor 5 (DR5) as a substrate of RNF183 using shotgun proteomic analysis. DR5 is known as a cell surface receptor of the TNF-receptor superfamily and it promotes TNF-related apoptosis inducing ligand (TRAIL)-induced apoptosis signal through interaction with caspase-8. We showed that RNF183 induced degradation of DR5 in the

lysosome through K63-linked ubiquitination. We also identified that RNF183 promoted TRAIL-induced activation of caspase-3 and caspase-8 through ubiquitination of DR5. Thus, RNF183 promoted not only DR5 transport to lysosomes but also TRAIL-induced caspase activation. Taken together, our findings suggest that RNF183 may play important roles in the onset and/or exacerbation of DSS-induced colitis and IBD through DR5 degradation.

P716/B733

Quantitative an alysis of the UPS Reveals Productive Adaptive Responses to Both Proteasomal and Protein Folding Stressors.

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Failures in the ubiquitin-proteasome system (UPS) are associated with challenges such as aging, disease-causing mutations, and environmental conditions. Yet a quantitative understanding of how the UPS responds to stress conditions is lacking. Here we characterized the performance and adaptability of the UPS in yeast using quantitative measurements of UPS performance (the ability to degrade misfolded substrates) and adaptation (transcriptional regulation of UPS components via the stress-sensing gene, RPN4). We observed multiple adaptive regimes, including perfect adaptation driven by two independent mechanisms: stabilization of the normally degraded Rpn4 protein under proteasomal stress and increased transcriptional of RPN4 under protein folding stress. Critically, we found that degradation-based regulation of Rpn4 is insufficient for UPS adaptation because protein folding stressors caused misfolded proteins to aggregate rather than become targeted to the proteasome and outcompete Rpn4 for degradation. Our work suggests the yeast proteasome evolved to favor aggregation of misfolded proteins over their degradation and reveals the versatility of cells in productively adapting to proteotoxic stress.

P717/B734

Novel Approaches to Protac Drug Discovery.

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Proteolysis targeting chimeras (PROTAC) is a very promising strategy to ubiquitylate and degrade target proteins, especially undruggable targets. Despite initial euphoria over this novel approach to knock down a protein function, meaningful progress has not been made. Nearly all the companies working to develop PROTAC drugs are focused on known ligases, such as cereblon, VHL or HDM ligases as vehicles. This approach may be flawed, however, in that nature does not employ the “one ligase fits all” target approach. In addition, major hurdles hinder current screening approaches, which include western blotting to monitor degradation of protein bands or reporter-based cellular assays such as that developed by Promega. These methods require multiple steps and are time-consuming, irreproducible, subject to human error, and not amenable to high throughput screening. Cell reporter assays require the generation of stable cell lines and are prone to artifacts. Moreover, current PROTAC screens do not provide any information regarding the poly-ubiquitination state of the target protein, a modification required for degradation. These problems have frustrated medicinal chemists and biochemists alike. We have developed a high-throughput microtiter plate-based approach, the UbiQuant Ultra, that evaluates protein ubiquitination, a true measure of a PROTAC molecule under physiological conditions. We have demonstrated the efficiency of a bromodomain PROTAC and a promiscuous kinase inhibitor that subsequently degrade their corresponding target proteins. We have established poly-ubiquitination and

degradation of BRD3 in response to bromodomain PROTAC in a microplate environment. These data correlate directly with gel-based analysis of PROTACs, and the method itself rapidly provides reproducible data, requires less tissue sample, and is easily translated to a plate-based environment, thereby remarkably enhancing PROTAC drug discovery. The application of unique affinity matrices -- our tandem ubiquitin binding entities (TUBE)-based mass spec proteomics platform -- allowed us to confirm the selectivity and degradation status of the target proteins in response to treatment with various PROTACs. For the first time, one can determine if a PROTAC ubiquitinates a target protein without degrading it, thus allowing chemists to design selective PROTACs. The goal of this study is to implement efficient methods to analyze PROTAC functions in a high-throughput fashion and accelerate development of novel PROTAC drugs.

P718/B735

Two Alternative Mechanisms Regulate the Onset of Chaperone-mediated Assembly of the Proteasomal Atpases.

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The proteasome holoenzyme is a molecular machine that degrades most proteins in eukaryotes. In the holoenzyme, its heterohexameric ATPase injects protein substrates into the proteolytic core particle, where degradation occurs. The heterohexameric ATPase, referred to as 'Rpt ring', assembles through six ATPase subunits (Rpt1-Rpt6) individually binding to specific chaperones (Rpn14, Nas6, Nas2 and Hsm3). Here, our findings suggest that the onset of Rpt ring assembly can be regulated by two alternative mechanisms. Excess Rpt subunits relative to their chaperones are sequestered into multiple puncta specifically during early-stage Rpt ring assembly. Sequestration occurs during stressed conditions, for example heat, which transcriptionally induce Rpt subunits. When the free Rpt pool is limited experimentally, Rpt subunits are competent for proteasome assembly even without their cognate chaperones. These data suggest that sequestration may regulate amounts of individual Rpt subunits relative to their chaperones, allowing for proper onset of Rpt ring assembly. Indeed, Rpt subunits in the puncta can later resume their assembly into the proteasome. Intriguingly, when proteasome assembly resumes in stressed cells or is ongoing in unstressed cells, excess Rpt subunits are recognized by an alternative mechanism—degradation by the proteasome holoenzyme itself. Rpt subunits undergo proteasome assembly until the holoenzyme complex is generated at a sufficient level. The fully-formed holoenzyme can then degrade any remaining excess Rpt subunits, thereby regulating its own Rpt ring assembly. These two alternative mechanisms, degradation and sequestration of Rpt subunits, may help control the onset of chaperone-mediated Rpt ring assembly, thereby promoting proper proteasome holoenzyme formation.

P719/B736

The Cdc48 Complex Alleviates the Cytotoxicity of Misfolded Proteins by Regulating Ubiquitin Homeostasis.

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Failure to destroy misfolded proteins is associated with cytotoxicity and is a hallmark of multiple neurodegenerative disorders. The mechanism(s) by which accumulation of misfolded proteins causes cytotoxicity remains poorly defined. Here, we demonstrate that the Cdc48/p97 segregase machinery drives clearance of the ubiquitinated model misfolded protein Huntingtin (Htt103QP) and limits its

aggregation. We show that the nuclear ubiquitin ligase San1 involved in protein quality control acts upstream of Cdc48 to ubiquitinate Htt103QP. Unexpectedly, we find that deletion of *SAN1* and/or its cytosolic counterpart, *UBR1*, rescues the toxicity associated with Cdc48 deficiency, raising the possibility that ubiquitin depletion, rather than compromised proteolysis, was responsible for the growth defect caused by Cdc48 deficiency. Indeed, Cdc48 deficiency leads to elevated protein ubiquitination but decreased free ubiquitin, which depends on San1 and Ubr1. We further show that enhancing free ubiquitin levels rescues the toxicity in various Cdc48 pathway mutants, and restores normal turnover of a known Cdc48-independent substrate. Our work highlights a previously unappreciated function for Cdc48 in ensuring the regeneration of monoubiquitin that is critical for normal cellular function.

P720/B737

Broad Inhibition of Deubiquitinases Unmasks Functional Redundancy Revealing Novel Proteasome Substrates and Enabling Specificity Profiling of These Enzymes.

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Deubiquitinating enzymes (DUBs) counteract substrate ubiquitination, affecting stability, activity or localization of the ubiquitinated proteins. The human genome encodes approximately 100 DUBs and most have no known substrates. Identifying DUB substrates and assigning specific biological functions to a DUB is challenging due to the fact that DUBs may act redundantly. Here, we circumvented the effects of redundancy by broadly inhibiting deubiquitination. We used quantitative mass spectrometry to identify proteins that are destabilized by broad DUB inhibition in *Xenopus* egg extract, and recapitulated this behavior with recombinant human proteins, demonstrating that their DUB-dependent stability is evolutionarily conserved. The majority of these proteins have not been reported previously as DUB substrates, even though some have been well-studied, such as the tumor suppressor Ing2, the Ewing tumor-associated antigen 1 (Etaa1) and the ubiquitin ligase Mkrn1 that targets p53. With a panel of novel substrates in hand, we tested the ability of individual DUBs to rescue degradation of these proteins. This approach revealed that USP7 has a broad ability to rescue these substrates from degradation. However, specific inhibition of USP7 was not sufficient to induce substrate instability, suggesting that DUBs function redundantly to control the stability of these proteins. Together, we present a new approach to identify DUB substrates and a system to characterize DUB specificity, overcoming the challenges posed by redundant action of DUBs.

P721/B738

Interactions between E3 Ubiquitin Ligases and Deubiquitylases - an Emerging Feature of Cellular Ubiquitin Dynamics.

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NEDD4 family E3 ubiquitin ligases, which are dysregulated in various human diseases, have been implicated as regulators of many membrane trafficking events in human cells, yet the mechanisms of substrate targeting and regulation remain poorly understood. We have identified specific deubiquitylating enzymes (DUBs) that interact physically with NEDD4 family E3 ligases to regulate specific ubiquitylation outcomes. Here, we describe recent experiments interrogating the regulatory functions of these E3-DUB interactions. We describe an E3-DUB interaction that governs a ubiquitin rheostat on a critical signal transducing protein in the WNT pathway. This rheostat influences toggling

between canonical and non-canonical states, thus impacting cellular behavior and response to environmental cues. These results underscore the dichotomous roles DUBs may play in the context of human cancer progression and have important implications for selection of DUBs as potential therapeutic targets.

P722/B739

Intracellular Regulation of Ate1 Isoforms.

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Arginyltransferase (Ate1) mediates posttranslational protein arginylation and plays key roles in multiple physiological processes, including cardiovascular development, cell migration, and cytoskeletal organization. This enzyme is represented in mammalian cells by four alternatively spliced isoforms, expressed from a single gene and previously proposed to play different roles in vivo. The molecular distinctions between Ate1 isoforms are not well understood. Moreover, very little is known about the regulation of Ate1 in cells. Here we investigate intracellular Ate1 regulation by examining its isoform-specific compartmentalization and metabolic fate. Our results suggest that Ate1 isoforms have different intracellular localization and appear to shuttle between the cytoplasm and the nucleus. Cell treatment with proteasome inhibitor MG132 leads to isoform-specific changes in Ate1 intracellular levels and distribution, and its accumulation as perinuclear aggregates. Further analysis suggests possible Ate1 regulation by ubiquitinylation and other post-translational modifications that may modulate its reversible association with different intracellular compartments. Our results provide new insights into the molecular differences that underlie the differential function of Ate1 isoforms.

Chemical Cell Biology

P723/B741

Using Resistance an alysis to Develop Chemical Probes for the AAA Protein Katanin.

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Achieving selectivity for a single protein within a family presents a major barrier to chemical probe design. We are developing an approach, Resistance an alysis During Design (RADD), for revealing key interactions with target proteins and guiding the design of selective probes. RADD involves identification of non-conserved residues in an active site (“variability hot-spots”) and mutation of these residues in the target protein to those found in other members of the protein family. An alysis of compound activity against the resulting mutant alleles informs on small molecule binding poses. However, our analysis has thus far been limited to one protein from the AAA (ATPases associated with diverse cellular activities) family and two chemical scaffolds. Here, we describe application of RADD to katanin, a microtubule-severing AAA protein proposed to regulate microtubules in spindles and cilia. We find that mutating katanin at its variability hot-spot residues (Leucine-214, Threonine-253, Alanine-419, and Threonine-422) does not abolish nucleotide binding or ATP hydrolysis activity. Testing a heterocycle-based small molecule that inhibits katanin at low micromolar concentrations against these mutant alleles indicates active site residues that interact with the inhibitor. These data guided the design of compounds with

~10-fold improvements in potency over the parent compound. Our results suggest the broader utility of RADD as an inhibitor design approach. Mutations characterized in this in vitro work could also be used for conferring resistance to the inhibitor in cellular studies. Katanin-specific phenotypes are identified as those modulated by inhibition of the wild-type protein, but not the mutant protein.

P724/B742

Metabolic Toxicity of Vespa Amino Acid Mixture and Other Amino Acid Mixtures in *Artemia Salina*.

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Vespa amino acid mixture (VAAM) is a solution of free amino acids secreted by larval Asian giant hornets - VAAM and similar solutions produced by other vespid species are thought to augment respiratory capacity. Previous work has shown increased ATP production is accompanied by destabilization of the inner mitochondrial membrane in mitochondrial isolates, cultured cells, and primary cell isolates of a variety of species. To evaluate the relationship between the specific amino acid profile of VAAM and mitochondrial metabolism, *Artemia salina* nauplii were assayed for ATP production, reactive oxygen species (ROS) production, and viability under serially diluted VAAM over a period of 24 hours. A mixture of amino acids replicating the hemolymph free amino acid composition of *A. salina* (HC) and a suspension of proline alone were used at matching concentrations as controls. Nauplii treated with 0.05% VAAM (or higher concentrations) showed a ≥ 53% decrease in viability after 24 hours relative to non-treated controls, while HC and proline treatments showed 10% and 6% reductions, respectively. ATP levels peaked at 1 hour for VAAM treated groups (2.5x control), while HC peaked at 12 hours (1.2x control) and proline at 24 hours (1.3x control). ROS production was negligible in non-VAAM treated groups, and peaked at 2 hours under VAAM treatment. Taken together, these data suggest that the mixture of amino acids in VAAM has a distinct interaction with cellular chemistry to influence mitochondrial function, and that the augmentation of aerobic respiration is not a result of increased reactant concentration.

P725/B743

Rational design of a chemically inducible t*rimerizaton s*ystem.

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In response to environmental cues many cell signaling events occur rapidly on the order of seconds to minutes. Chemically inducible dimerization (CID) strategies such as the FKBP - FRB system can heterodimerize proteins of interest (POIs) inside cells at a similar timescale upon addition of small molecule drug rapamycin. CID is commonly used to translocate POIs towards or away from desired subcellular locations by using protein localization tags and can elucidate local protein function or synthetically actuate or perturb a downstream event. Other similar systems using chemical or light stimulation also cause dimerization or homo-oligomerization. However, no inducible trimerization system has ever been developed. We engineered a rapamycin-dependent system which we term chemically inducible trimerization (CIT). To our knowledge, this is the first such system which brings together three distinct proteins, perhaps because a trivalent chemical approach confers an extra degree of freedom and is thus challenging. With computational aid, we rationally designed split sites for FKBP and FRB. Positive split sites were identified by ability of plasma membrane (PM)-localized split FRB

(sFRB) and FKBP (sFKBP) pairs to recruit FKBP or FRB respectively upon rapamycin administration. Trimerization still occurred when sFRB or sFKBP were localized to different places in the cell, indicating that proximity is not a limiting factor in split pair reconstitution. In-cell kinetics of FKBP recruitment by an sFRB pair was determined, with exponential recruitment rate coefficient of -0.055 ± 0.01 1/s compared to full length FRB at -0.076 ± 0.01 1/s. Using CIT, cytosolic proteins could be conditionally targeted to inter-organelle membrane contact sites such as ER-PM and ER-mitochondria junctions. ER-PM junctions can be sites of lipid transfer and metabolism, which includes the regulation of $PI(4,5)P_2$ (or PIP2). While translocation of phosphatases or kinases to the ER or PM is possible with CID to perturb PIP2 levels, translocation contingent upon both ER and PM presence has not been achieved. Using CIT, we recruited PIP2 phosphatase INP54P to ER-PM junctions and locally depleted PIP2 at these sites - far beyond recruitment of FKBP alone or phosphatase-dead versions. This suggests that CIT can be used as a general paradigm to perturb ER-PM and other junctions. Altogether, we combined concepts of inducible dimerization and rational protein splitting to generate an inducible hetero-trimerization system, which can be useful for probing biological questions requiring three signals.

P726/B744

Bioorthogonal Smart Probe for Fluorescent Imaging of Carbonyl Moieties in Live Cells.

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In recent years, bioorthogonal chemistry has found significant use in medical sciences. Controlling stable chemical reactions in biological systems by using bioorthogonal chemistry is not only one of the powerful tools but also the most challenging method, which has been widely performed with synthetic chemical probes to tag biomolecules in live cells. Site-selectively tagging and controlling biomolecules with suitable probes inside cells has the potential to manage challenges faced in clinical practice for early diagnosis and treatment of tumors. Herein, we designed a hydrazine based small molecule, which incorporates with the carbonyl moiety of biomolecules through click reaction to form a fluorescent hydrazone product, therefore can be utilized for the detection of biomolecule carbonylation in various cancer cell lines. Our almost non-fluorescent chemical probe can make fast covalent binding with carbonyl moieties at neutral pH to form a stable product leading to a red shift on the absorption maximum and an increase on the fluorescence quantum yield. Microscopic and fluorometric analyses were used to distinguish the exogenous and endogenous ROS induced carbonylation profile in human dermal fibroblasts along with A498 primary site and ACHN metastatic site renal cell carcinoma (RRC) cell lines. Confocal image analysis, UV-vis and spectrofluorometric measurements were also performed to detect and characterize the bioorthogonal coupling reactions. Our results showed that carbonylation level that differs in response to exogenous and endogenous stress in healthy and cancer cells, can be monitored by the newly synthesized chemical probe. When cells were exogenously ROS induced, A498 cell line demonstrated higher carbonylation level than the ACHN cells. Our results will therefore expand the applications of novel smart probes that are expected to enable deeper and more selective studies of cellular systems to enhance new diagnostic approaches in cancer.

P727/B745

Imaging Neuron Activity-dependent Compartment-specific Redox Dynamics.

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Reactive oxygen species (ROS) are integral components of redox signaling in cells and mediate physiologically essential signaling across compartments. Under pathological conditions, excessive presence of ROS can lead to oxidative stress that damages cellular components and leads to cellular dysfunction and death. To understand dynamic redox changes and identify the mechanisms that disrupt redox homeostasis, we need high-precision tools that can be used to define redox processes in live cells under physiological and pathophysiological conditions. Genetically encoded fluorescent sensors that measure cellular redox status can be targeted to specific subcellular compartments to study the redox changes in intracellular compartments during live-cell imaging. However, it has remained technically challenging to quantitatively correlate spatially distinct redox dynamics with subcellular resolution. To address this challenge, we developed red fluorescent redox sensors that can be multiplexed with reduction-oxidation-sensitive green fluorescent proteins (roGFP) to image dual compartments within the same cell. Our approach utilized Förster-type resonance energy transfer (FRET) in a spectral relay strategy that extends the fluorescence emission of roGFP into red emission wavelengths We targeted the redox sensors to mitochondria and cytosol in primary hippocampal neurons and monitored sensor response to oxidizing and reducing agents. Upon neuronal stimulation of the neurons with glutamate, we observed an increase in mitochondrial oxidation accompanied by a cytosolic reduction. Pre-treatment with antioxidants MitoTEMPO and N-Acetyl Cysteine attenuated both the neuron-activity dependent cytosolic reduction and mitochondrial oxidation implying that these redox changes were coupled. In a mixed culture system, astrocytes are known to have a neuroprotective effect from oxidative insults and glutamate excitotoxicity. We targeted the redox sensors in a neuron-astrocyte co-culture system to observe the neuroprotective effect of astrocytes on neuronal redox dynamics. Using this sensor in combination with the roGFP, we can conduct live-cell imaging with spatio-temporal accuracy to delineate sub-cellular changes in redox dynamics that result in pathological oxidative stress.

P728/B746

The Effect of the Antioxidant Ascorbic Acid on the Energy of Activation of Free Radical Inhibition.

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It is widely accepted that free radical damage is involved in the development of numerous human pathologies, such as Alzheimer's and Parkinson's diseases, atherosclerosis, and some cancers. Thus, we previously developed a chemical model system utilizing a hydrogen peroxide-initiated luminol reaction to evaluate the efficacy of antioxidants to inhibit free radical-induced chemiluminescence. This chemiluminescence is proportional to the number of diradical intermediates produced in the reaction sequence. Thus, the purpose of the present study is to utilize this chemical model system to evaluate the effect of the antioxidant ascorbic acid on the energy of activation of free radical production. All reactions were run in at least duplicate or triplicate at pH 7.2 using a sodium phosphate buffer. First, a dose response for the inhibition of chemiluminescence was demonstrated utilizing ascorbic acid at concentrations of 0, 21, 42, and 85 µM, respectively. Then, a dose response was established for the inhibition of chemiluminescence by ascorbic acid at temperatures of 23, 30, 37, and 42 ^{o}C, respectively, for the baseline luminol reaction. In these reactions no ascorbic acid was present. This was followed by a series of luminol reactions run at each of these temperatures, respectively, for concentrations of

ascorbic acid of 0, 42, and 85 µM, respectively. From these luminometry data, the respective rate constants were determined, so as to allow an Arrhenius plot for the luminol reactions run with 0, 42, and 85 µM ascorbic acid, respectively. Thus, it was determined that in the absence of ascorbic acid, the energy of activation for the luminol reaction was 39.9 joules/mole, whereas the energy of activation was 131.14 joules/mole with 42 µM ascorbic acid and 168.94 joules/mole when the concentration of ascorbic acid was increased to 85 µM in the reaction system. Thus, as expected, the data clearly show that as the temperature of the luminol reactions was increased, both the rate and extent of reaction product increased for each of the above reaction series. However, these data also clearly demonstrate that the antioxidant ascorbic acid increases the energy of activation of the free radical-producing reactions they inhibit. This provides additional insight into the mechanisms by which antioxidants inhibit free radicals, and suggests that it is likely a similar result would be obtained with other antioxidants, such as NADPH, glutathione, and resveratrol. Current experiments are underway to test this hypothesis. Finally, the manner in which these experiments are designed could certainly provide an efficacious model that could be adapted into a number of informative and unique biochemistry/cell biology laboratory exercises.

P729/B747

Highly Selective in *Vivo* Editing of Macrophages through Systemic Delivery of Crispr Cas9-Ribonucleoprotein-nanoparticle Nanoassemblies.

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Gene editing of macrophages is a promising therapeutic strategy due to their role in host defense systems. Administration of protein-based CRISPR therapeutics in vivo is challenging due to proteolysis and endosomal entrapment. Previous work in our lab has demonstrated supramolecular nanoparticle-protein delivery assemblies for direct intracellular delivery, of recombinant CRISPR/Cas9 machinery. We hypothesized that systemic introduction of these nanoassemblies would provide efficient gene editing in *vivo* as well. Intravenous administration of CRISPR/Cas9 nanoassemblies into mice achieved specific localization and effective gene editing in macrophages. Through this approach, >8% gene editing efficiency specifically in macrophages of the liver and spleen was observed, with no detectable off-target editing. These systems allow the capability to engineer phagocytes *ex vivo* and in *vivo*, and thus could be an effective and very appealing approach to the development of immunotherapeutics (Ray, et al. 2018).

P730/B748

Sequence Determinants in the Allosteric Control of Camp Receptor Protein.

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In this study, we aim to define the biophysical principles by which two functionally and structurally conserved proteins are allosterically activated differently, and how the results challenge the “structure-function” paradigm. The homodimeric cAMP Receptor Protein (CRP) is a bacterial transcription factor that is allosterically activated by cAMP. The cAMP-bound conformations of CRP from *Escherichia coli* ($CRP_{E.coli}$) and from *Mycobacterium tuberculosis* (CRP_{MTB}) are nearly identical, but their unbound conformations display significant differences, indicating that the allosteric regulation of these homologs do not follow the same mechanism. Previous studies of $CRP_{E.coli}$ have shown that interdomain communication across the dimer interface or between cAMP- and DNA-binding domains are critical to

$CRP_{E.coli}$'s function and allosteric regulation. However, the allosteric activation mechanisms of CRP_{MTB} are less known. To understand the importance of individual residues in the allosteric regulation of CRP_{MTB}, we made five single-point mutants based on well-characterized mutations in $CRP_{E.\ coli}$ that have been shown to modulate cAMP and DNA binding affinities. All the mutations were found to have comparable thermodynamic stability and secondary structure compared to wild-type CRP_{MTB}. However, cAMP and DNA binding assays show that mutational effects in $CRP_{E.\ coli}$ are non-translatable to CRP_{MTB}, indicating that single residues alone may not be the determining factor of allosteric communication. In addition to single mutants, we use ancestral reconstruction of CRP family members to introduce a collection of mutations to the protein. We find that the ancestral $CRP_{E.\ coli}$ and the ancestral CRP_{MTB} display similar folding and cAMP binding characteristics to their respective extant proteins. This may suggest that multiple amino acid residues are responsible for specific allosteric behavior, and that allosteric mechanisms cannot be tuned alone through single-site mutations. A common ancestor of both proteins will reveal the conserved motifs that give rise to the observed differences in allosteric regulation of CRP between *E. coli and MTB.*

P731/B749

Reactive Oxygen Species Generation in Plant Protoplast Triggered by Herbivore Oral Secretions.

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Plants are under constant attack by a suite of herbivorous insects and consequently, they have evolved a wide range of counter-defense strategies to either manage or resist them. These include physical structural defenses such as trichomes and spines and chemical defenses such as toxic secondary metabolites. Plant-herbivore interactions mediated signaling are generally initiated at the plant protoplast membrane, where herbivores physically damage the plant. Herbivore oral secretions (OS) trigger a series of signaling cascades that eventually lead to the mounting of various plant defense mechanisms. Reactive Oxygen Species (ROS) is a signaling molecule that has been known to play a role in activating plant defense cascade at the cellular level. While behavioral and mechanistic layers of such interactions have been well understood, the physiological link between the plant ROS production and herbivory is less understood. Here, we evaluated the effect of herbivore tobacco hornworm caterpillar (*Manduca sexta*) OS on the ROS level in isolated tomato (*Solanum lycopersicum*) protoplasts. Using the dye-based ROS imaging technique, we found that the Plant-Fed (PF)-*Manduca* OS triggered ROS production while artificial Diet-Fed (DF)-*Manduca* OS failed to induce ROS in tomato protoplasts. The addition of antioxidant NAC (N-acetyl-L-cysteine) antagonized the PF-*Manduca* OS induced ROS generation. Interestingly, Ca^{2+} chelator BAPTA-AM attenuated ROS induction by PF-*Manduca* OS, suggesting possible crosstalk between Ca^{2+} and ROS signaling. Taken together, our findings indicate that herbivores OS can directly modulate cellular ROS production and possibly regulate defenses against insect herbivores.

P732/B750

Role of an ionic Polypeptides in Lysozyme Phase Separation.

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Membrane-less organelles formed via liquid-liquid phase separation of biopolymers plays an important role in spatial organization of cytoplasm and nucleoplasm. Most of the cellular condensates are highly enriched with proteins containing intrinsically disordered regions. This is related to the fact that the flexible intrinsically disordered regions can easily form multivalent interactions with other chains with consequent phase separation. Conversely, it is believed that membrane-less organelles assembly by structured proteins is uncommon due to the high concentrations required for phase separation of such proteins. We suggest that the presence of highly oppositely charged disordered polypeptides which is wide-spread in proteome may reduce the threshold concentration of ordered proteins phase separation making it a general phenomenon in cells. In the present work pH dependence of the effect of anionic polypeptides on phase separation of the basic ordered protein lysozyme was studied. Poly-glutamic acid with polymerization degree 50 and intrinsically disordered protein prothymosin α, containing >30 % of negatively charged amino acids were used as anionic polypeptides. Concentration of polyanions and lysozime was 0.2 mg/ml 5 mg/ml, respectively. Using static light scattering measurements, we showed that at 2.5 < pH < 6.5 and 8.0 < pH < 10.4 homogeneity of lysozyme solutions was disturbed by injection of both polyanions. Examination of the turbid solutions using differential interference contrast microscopy showed that with the exception of macromolecular associates assembled at pH 5.0, all formed aggregates were amorphous. At pH 5.0 mixing of lysozyme with prothymosin α or poly-glutamic acid results in liquid-liquid phase separation and crystal formation respectively. Presumably, the more efficient screening of positively charged surface clusters of lysozyme by poly-glutamic acid leads to protein dehydration and drive the complex directly to solid crystals formation. Interestingly, the presence of poly-glutamic acid reduces the lysozyme concentration required for crystallization by at least factor four. Such effective salting out of ordered proteins by oppositely charged polypeptides in cells may induce membrane-less organelles formation involving even ordered proteins as scaffolds. Our results suggest, that both studied polyanions: prothymosin α and poly-glutamic acid, exhibit the same effect of lysozyme salting-out, to a different extent. This work was supported by the Russian Foundation of Basic Research (grant numbers 18-34-00975), and the RF President Fellowship (number SP-259.2019.4).

Physical Approaches to Cell Biology

P733/B751

Dynamic Crosslinking of the Actin Cytoskeleton Governs Cell Mechanics.

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Precise control of the cell's mechanical properties is required for cells to organize and differentiate into tissues and organs. The architecture and connectivity of cytoskeletal filaments changes in response to mechanical and biochemical cues, allowing the cell to rapidly tune its mechanics from highly-crosslinked, elastic networks to weakly-crosslinked, viscous networks. Here, we probe the frequency-dependent

viscoelastic properties of the cytoplasm in living cells using multiharmonic excitation and in *situ* optical trap calibration. At long timescales, we observe pronounced fluidization of the cytoskeleton. The mechanical response is well captured by a model where actin filaments are connected by a single dominant crosslinker. A disease-causing point mutation (K255E) of the actin crosslinker α- actinin 4 (ACTN4) renders it insensitive to tension. We show that under normal conditions, the viscoelastic properties of wild type (WT) and K255E+/- cells are similar. However, when tension is reduced through myosin-II inhibition, WT cells relax 3x faster while K255E+/- cells are not affected. Our results show that the cytoplasm behaves like a fluid at long timescales and suggest that dynamic actin crosslinking regulates relaxation.

P734/B752

Microfluidic Guillotine Reveals Multiple Mechanisms and Time Scales of Single-cell Wound Healing.

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Wound healing is a key feature of living systems. At the single cell level, the ability to heal wounds underpins fundamental biological processes from cytokinesis to cancer metastasis. For some organisms, wound healing can occur in two stages: short-term sealing of the plasma membrane opening, and long-term regeneration to rebuild damaged or lost structures. While several model organisms have been developed to study single-cell wound healing, many open questions remain regarding the mechanisms and dynamics of single-cell wound healing. Here we describe a microfluidic “guillotine” as a platform to probe the mechanisms and time scales of single-cell wound healing using *Stentor coeruleus*, a giant ciliate, as our model. In this study, cells were bisected in a microfluidic guillotine, which is a knife blade fabricated inside a microfluidic channel. The cells are introduced into the guillotine at various flow rates using a syringe pump. Varying the flow rate yields two distinct wounding regimes of the guillotine (a low viscous stress Regime I and a high viscous stress Regime II). To quantify the extent of wound repair, we fixed the cells at selected time points after wounding and stained them with Sytox Green, a membrane impermeable dye. Using this assay, we found that Regime II wounds are larger, require longer repair times, and reduce survival rate compared to Regime I. Surprisingly, we observe in Regime II that after the cell is cut, the wound appears to first increase in size before closing. As an alternative wounding method, we performed laser ablation experiments on the cell membrane, and the results were consistent with both the membrane sealing time scale and the apparent increase in wound size observed using the microfluidic guillotine Sytox Green assay. Further, the survival rate derived from the Sytox Green assay agrees with the survival of observed cells over 24 hours. Finally, we highlight a few examples of using the guillotine to probe mechanisms of wound repair and regeneration. Since Ca^{2+} ion influx is a known initiator of wound repair, we confirmed that using a reduced Ca^{2+} media inhibits wound repair. Dynein inhibition by $NiCl_2$ treatment results in improper morphology at 24 hours, possibly due to the inability for the cell to reestablish polarity. Protein synthesis inhibition by cycloheximide treatment results in various defects at 24 hours, likely because regeneration requires the synthesis of new proteins to rebuild cell structures. Currently, it is unknown whether *Stentor* use the same pathways to heal wounds as classical model organisms do. We plan to test these mechanisms in *Stentor* using RNAi and drug treatments. We expect to further use the microfluidic guillotine and wound healing assay presented here to answer key questions on single-cell wound healing.

P735/B753

Nanoscale Structural Dynamics of the Cell Wall during Pole-growth in Fission Yeast.

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The rod-shaped eukaryote fission yeast is a widely used organism to study general principles governing the regulation of cell size, growth and morphogenesis. Fission yeast cells are inflated by a high turgor pressure which is counter-balanced by a rigid cell wall. Maintenance of the cell wall structural integrity while allowing remodelling of the cell wall for the cell to grow is key to survival. The cell wall is a complex structure composed of the glucose polysaccharides α-, β-glucan and glycoproteins and defines the shape of the cell. During periods of growth, new cell wall material is inserted at the cell poles, subsequently forming the cylindrical part of the cell as the pole continues to insert new wall material displacing existing material. The micron-scale cell shape and growth patterns are orchestrated by the nanoscale structural and mechanical cell wall dynamics. However, the mechanisms linking these phenomena are unknown. To study the dynamical changes of the cell wall during fission yeast growth we use time-lapse live cell atomic force microscope (AFM) imaging on poles of vertically immobilized cells. High-resolution imaging reveals the dynamic rearrangement of glucan fibers on the pole as the new pole initiates growth after new end take off (NETO) and glucan strands are being incorporated into the cell wall. The nanoscale structural rearrangement in conjunction with local mechanical properties provide unprecedented detail of the pole-growth process highlighting the contribution of the cell wall stress on incorporation and dynamical rearrangment of glucan fibers. In addition we'll characterize the role of specific steps of the cell wall biosynthetic pathway on the nanoscale cell wall structure and dynamics and establish its effects on cell shape changes. This will provide the structural and mechanical details for establishing general principles linking the cell wall rearrangement at the nanoscale to the emergence of micron-scale cell shapes.

P736/B754

Characterizing the Effect of Nick and Holiday Junction on Axial Stiffness of DNA Origami.

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The techniques of DNA origami have been applied to build nano-sized structures of a variety of geometries, ranging from rectangular boxes intended for drug delivery to robots capable of dynamic displacement. Nevertheless, the mechanical property of the DNA origami structures still remains unclear, making it difficult to predict their long-term functionality. Here, we aim to understand whether the structures of DNA origami, nick and Holliday Junction, affect its axial stiffness. Nick, the region where one staple strand ends and another begins, and Holliday Junction, the region where a staple strand extends from one double-helix bundle to another. The axial stiffness of four DNA origami structures in the shape of beams, with different number of nicks and Holliday Junctions, were examined by applying pulling forces to stretch the DNA origami using a microfluidic device. The DNA origami was immobilized on a glass surface, and stretching forces ranging from 1 pN to 30 pN were applied to the DNA origami through a micron-sized particle bound to the free end of the DNA origami. The measurement showed a 1.3-fold higher axial stiffness in the ligated DNA origami structures than the unligated ones, suggesting the presence of nick reduces the axial stiffness of DNA origami. In parallel, we also observed that reducing the number of Holiday Junctions by 50% increased the stiffness by 1.6-fold, suggesting that

Holliday Junctions weaken the structural integrity, thus decrease the stiffness of DNA origami. Our finding can provide useful rules when designing the DNA nanostructure with desirable mechanical properties.

P737/B755

Protein Condensates Are Aging Maxwell Fluids.

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Liquid-like protein condensates (LLPCs) are intracellular compartments that segregate material without the use of a membrane. The liquid-like behavior of the condensates is a defining characteristic and the viscosity, surface tension and other material properties determine how segregated species diffuse into and within condensates; they, thus, critically impact the biological function of the condensates. It has become increasingly clear that some LLPCs do not have time-independent material properties, but can, instead, transition to more solid, gel-like materials. Here, we present our efforts to quantify these new materials as they age in vitro. We measure the visco-elastic material properties of two proteins, PGL-3 and FUS, by means of a combination of active and passive microrheology. At early times, we find that the droplets behave much like simple liquids but gradually become more elastic. Surprisingly, the changing mechanical properties can all be scaled onto a single master curve using one characteristic time scale which grows as the sample ages. This and other features we observe bear a striking resemblance to the behaviors observed in materials with glass-like aging. We conclude that protein condensates are soft glassy materials with age dependent material properties that we call Maxwell glasses. We speculate on the advantages of glassy behavior for modulation of cellular biochemistry.

P738/B756

A Versatile Platform for Measuring Cellular Forces in Complex 3d Environments.

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We have developed a method for measuring cellular forces within user programmable, 3D micropatterned environments. Our method uses flat or patterned polyacrylamide gels, coated in fibronectin as a substrate for cells to grow and migrate on. We control the topography of these gels by casting them on photolithographically patterned silicon wafers. With this approach, we demonstrate the ability to guide cell migration and measure cell forces within channels of various sizes that contain branches or constrictions at specified locations. We control the substrate stiffness by varying the ratio of acrylamide and bis-acrylamide as in (Tse and Engler 2010) and calculate cellular tractions by feeding the hydrogel rigidity and geometry into custom scripts in Matlab and Comsol. We are using these approaches to study the coordination between cytoskeletal dynamics and cellular tractions as human osteosarcoma cells transit through structures that mimic constrictions encountered at tissue interstices. We further demonstrate the versatility of this platform using both high-speed 3D lattice light-sheet microscopy and readily available wide field microscopes. Together, we anticipate that these methods will enable new studies of how cellular forces drive migration through a variety of complex and physiologically relevant 3D architectures.

P739/B757

Dynamically Heterogeneous Plasma Membrane Is Poised for Initiation of Receptor-mediated Mast Cell Signaling.

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Cell surface receptors distinguish specific stimuli from biological noise, and underlying mechanisms depend on the resting steady-state organization of plasma membrane components. The nanoscale distribution of signaling proteins, including kinases and phosphatases, is coordinately rearranged within the poised membrane after the receptor is stimulated by extracellular ligand, such that changes in relatively weak interactions serve to transmit the signal across the membrane. Central to membrane organization are dynamically heterogeneous features such as cortical actin meshwork and asters, ordered proteo-lipid nanodomains, and protein clusters, any or all of which may slow the diffusion of membrane components. Correspondingly, microscopic diffusion of structurally and functionally distinct components provide the means for examining nanoscale organization in resting and stimulated cells. We evaluated three key components in the early stage of mast cell signaling by measuring their diffusion in live RBL cells: FcεRI receptor for immunoglobulin E (IgE), tyrosine kinase Lyn anchored to the membrane inner leaflet, and transmembrane phosphatase PTPα. We exploited the statistical robustness of multiplexed Imaging Fluorescence Correlation Spectroscopy (ImFCS), initially to evaluate subtle diffusion differences of these proteins in resting cells. We found that interactions between its saturated lipid anchor and Lo-like lipid nanodomains govern the diffusing distribution of Lyn, which also undergoes protein-based interactions. Diffusion of transmembrane proteins, IgE-FcεRI and PTPα appears limited primarily by protein-based interactions. Antigen-crosslinking of monomeric IgE-FcεRI causes their nanoclustering and consequent stabilization of a locally ordered membrane environment. ImFCS data show that this subtly reorganized region acts as a spatial filter to facilitate co-localization of Lyn through its favorable lipid-based interactions and, simultaneously, segregation of PTPα based on unfavorable interactions of its transmembrane domain with ordered lipids and protein-based steric exclusion. Supporting this view, a Lyn chimera with unsaturated lipid anchor does not couple with antigen-crosslinked IgE-FcεRI, whereas a PTPα anchored to the membrane by saturated lipids does couple. These observations underscore the pivotal importance of organized plasma membrane heterogeneity in the poised resting state, thereby enabling orchestration of critical protein-protein interactions during stimulated transmembrane signaling. This research is supported by NIH R01GM117552.

P740/B758

Physical Mechanisms for Oncogene-induced Breakdown in Mammary Tissue Structure during Cancer Progression.

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Structural breakdown in the mammary epithelium is the hallmark of progression from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC), and represents a major inflection point in the risk for patients. Normal epithelium is comprised of an inner luminal (LEP) compartment, also the site of origin for most breast cancers, that is surrounded by an outer myoepithelial (MEP) layer. Therefore, we propose that translocation of transformed LEP past the MEP layer is a key rate-limiting step preceding invasion, as most breast cancer drivers are already activated in DCIS lesions. We previously

demonstrated that reconstituted organoids containing normal human LEP and MEP (isolated from reduction mammoplasty tissue) can self-organize in vitro, and that the capacity of MEP to exclude LEP from the basal compartment is determined by hard-wired and lineage-specific interfacial tensions at each cell-cell and cell-extracellular matrix (ECM) interface. Specifically, the LEP-ECM interface is highly unfavorable compared to the MEP-ECM interface. We developed a model for self-organization driven by differences in interfacial energy, and predicted that altered interfacial tensions and dynamics (motility) promote basal LEP translocation, downstream of many cancer genes. Specifically, our model predicts that stabilization of the LEP-ECM interface and increased dynamics will increase occupancy of a high energy structural intermediate with a LEP in the basal compartment. To test this, we expressed twelve common breast cancer drivers in normal LEP, and observed that only PIK3CA-H1047R and PIK3CA-E545K expression disrupted their organization with normal MEP in 3D culture. Consistent with our predictions, PIK3CA activation increases the LEP-ECM contact angle (a measure of favorability of the LEP-ECM interface), promotes tissue dynamics and upregulates expression of several ECM molecules. Further, we used chemical inhibitors to show Akt activation downstream of PIK3CA is necessary for tissue disorganization. Collectively, these results show that signaling downstream of PIK3CA, a pathway dysregulated in >70% of breast cancers, drives the physical changes necessary for the structural transition that is a prerequisite for invasion. A more mechanistic understanding of the physical and molecular changes driving this transition will benefit the patients currently being overtreated due to lack of good markers for IDC progression.

P741/B759

Deciphering Mechanisms of Lipid Droplet Biogenesis.

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Lipid droplets (LDs) play a central role in cellular energy balance and are implicated in several biological processes. They are unique organelles as they are made by a neutral lipid core covered by a phospholipid monolayer, while other cellular organelles have a bilayer. LD biogenesis occurs at the endoplasmic reticulum (ER) phospholipid bilayer. During lipid storage conditions, neutral lipid oil molecules synthesized in the ER are encapsulated in between the monolayers composing the ER bilayer. At a critical concentration, the free neutral molecules demix and phase separate from the bilayer to nucleate a droplet. This nucleation step requires to cross an energy barrier which likely depends on membrane biophysical parameters such as tension, curvature or physicochemistry. However, it is unclear how these parameters impact LD nucleation from a bilayer. We opted for an in vitro reconstitution approach to study processes of LD nucleation. We incorporated neutral lipids into giant unilamellar vesicles to reconstitute situations prior LD nucleation. This system enabled to modulate membrane physicochemical and biophysical properties so as to depict parameters critical for phase separation of the neutral lipids and nucleation of a droplet. We found that membrane topology and tension control neutral lipid distribution in a bilayer. Indeed, we observed differential enrichment of neutral lipids between planar and tubular membrane regions. This led us to identify that membrane curvature modulates the energy barrier for nucleation and plays a key role in LD formation in a bilayer. In cells, several proteins have been reported to play key roles in LD nucleation. Our in vitro approach reveals that proteins are rapidly recruited to nucleated droplet and stabilize them by preventing their disappearance. Our approach for studying these early steps of LD biogenesis facilitated the identification of key parameters acting during this process. Clearly, its incrementation by the incorporation of more relevant membrane proteins will enable to address and better understand key questions of LD biology.

P742/B760

Nuclear Bodies Undergo Heterogeneous Nucleation Near the Binodal Line in Mitosis.

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Living cells utilize compartmentalization to concentrate certain biological molecules (protein, RNA, and lipids). The compartmentalization is carried out not only through membranes, which are composed of lipids bilayers and proteins, but also by liquid-liquid phase separation, which leads to membrane-less organelles or condensates. The membrane-less organelles or condensates disassemble and reassemble in mitosis during cell cycle; however, the biophysics of the process is poorly understood. Taking advantage the optoDroplet system we recently developed, we demonstrate that membrane-less organelles or condensates in nucleus (nuclear bodies)undergo heterogeneous nucleation near the binodal line in mitosis. Our findings give energetic understanding on how the cells spatio-temporally control the nucleation during cell cycle.

P743/B761

Mechanotransduction and Migration of *Dictyostelium on* Substrates of Different Stiffnesses.

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The stiffness of adherent mammalian cells is regulated by the elasticity of substrates due to mechanotransduction via integrin-based focal adhesions. *Dictyostelium discoideum* is an ameboid protozoan organism that does not carry genes for classical integrin and can adhere to substrates without forming focal adhesions. It also has a life cycle that naturally includes both single-cellular and multicellular life forms. We report the measurements of the elastic modulus of single cells on varied substrate stiffnesses and the elastic modulus of the multicellular “slug” using atomic force microscopy (AFM) as a microindenter/force transducer. The results show that the elastic modulus of the *Dictyostelium* cell is regulated by the stiffness of the substrate and its surrounding cells (Wu, Y and Cooper, KM, 2019). We also investigate the migration of the cells on substrates of different stiffnesses.

P744/B762

Extracellular Potential Measurement of Heart Tissue Pieces for Cardiotoxicity Testing Technology.

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Arrhythmia in the heart is one of the fatal diseases for the living body. Development of drugs in which arrhythmia may appear as a side effect may be stopped, and safety assessment is required at an early stage of development. As a means for solving this problem, Multi-Electrode Array (MEA) system is attracting attention. The MEA system is a device that measures the extracellular potential of cells and tissues present on MEA electrodes. The extracellular potential waveform of cardiomyocytes consists of peaks due to the inflow and outflow of three types of ions, Na^+, Ca^{2+}, and K^+. The potential from the Na^+ peak to the K^+ peak is the Field Potential Duration (FPD). It is used as an index corresponding to the QT interval. In addition, Short Term Variability (STV), which is a fluctuation including temporal factors, was calculated for FPD. In this study, a heart tissue piece derived from a chick embryo is placed on electrode,

its extracellular potential is measured. The cardiotoxicity of the drug is evaluated using E-4031, which is known to cause QT prolongation. We verified whether this is possible. The cardiotoxicity of 0.1 nM-1 μM E-4031 was verified in a 37°C incubation box and a CO_2 incubator. As a result, similar to the results with the previous human ES cell-derived cardiomyocyte mass, the addition of 1 μM E-4031 prolongs the FPD. E-4031 has the effect of specifically suppressing the K^+ channel, and it was confirmed that the K^+ peak of the waveform gradually decreased at 1 μM E-4031. In the CO_2 incubator, the STV of FPD was about 6 times that before the addition of E-4031 at 100 nM although FPD did not change. Previous results from human ES cell-derived cardiomyocyte clusters suggest that there is a possibility of cardiotoxicity when the STV value exceeds 1.9 times that before E-4031 addition. In this study, extracellular potential measurement using chick-embryo-derived heart tissue pieces detected cardiotoxicity at 100 nM, which has been reported to inhibit K^+ channels in existing in vitro cardiotoxicity tests. Therefore, it was suggested that CO_2 incubator experiments using chick-embryo-derived heart tissue pieces are useful.

P745/B763

Rigidity Dependent Spontaneous Epithelial Tissue Rupture.

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Epithelial integrity is a primary tissue function which protects the organs from abrasion and environmental exposure. Instability or gaps in this protective epithelial layer is a rare event. Studies in intestinal epithelium have shown gaps in the tissue after an apoptotic event[1]. Remarkably gaps in the epithelial have been observed upon mechanical load in lung epithelium[2] or in drosophila wings upon the loss of cell-cell adhesion[3]. However, the mechanism of this gap formation is still unclear. In our 2D in-vitro epithelial culture system (MDCK) we observe spontaneous gap formation in a substrate rigidity dependent manner. The size of these gaps can vary from 1-10 cell size. These gaps are able to seal themselves below a maximal gap size of about 4000μm^2. We observed two sources of gap formation: 1) from an edge of a stretching cell causing it to detach from its neighboring cell and 2) After cytokinesis and failure of complete sealing between the two daughter cells. We also observe that these gaps are formed in regions of high isotropic tensile stress characterized by -1/2 nematic defects. In the first case when cells get stretched prior to gap formation, we notice a peak in Von-Mises stress 30-60 minutes prior to gap formation. Von-Mises stress a term borrowed from material science determines the ductile to brittle yielding of a material. This has also been shown to be a leading cause of tissue rupture in *Trichoplax adherens*[4]. References 1. Watson, A. J., Duckworth, C. A., Guan, Y. An d Montrose, M. H. Mechanisms of Epithelial Cell Shedding in the Mammalian Intestine and Maintenance of Barrier Function. An nals of the New York Academy of Sciences, 2009, 1165: 135-142. doi:10.1111/j.1749-6632.2009.04027.x 2. John N. Maina, Sikiru A. Jimoh. Structural failures of the blood–gas barrier and the epithelial–epithelial cell connections in the different vascular regions of the lung of the domestic fowl, Gallus gallus variant domesticus, at rest and during exercise. Biology Open 2013 2: 267-276; doi: 10.1242/bio.20133608 3. An ne-Kathrin Classen, Kurt I. An derson, Eric Marois, Suzanne Eaton. Hexagonal Packing of Drosophila Wing Epithelial Cells by the Planar Cell Polarity Pathway, Developmental Cell, Volume 9, Issue 6, 2005, Pages 805-817, ISSN 1534-5807,

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P746/B764

Towards Structural an alysis of the Intracellular Pathogen Response Pathway in *Caenorhabditis Elegans*.

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Pathogen infection can perturb protein homeostasis (proteostasis) of the host. Therefore, organisms have evolved defensive mechanisms to sense pathogens and mount a protective response. For infections caused by microsporidia and viruses in *C. elegans*, the intracellular pathogen response (IPR) pathway is important for maintaining proteostasis. In the IPR pathway, PALS-25 and PALS-22 act as positive and negative regulators of genes induced by microsporidia and virus infection, including cullin-RING ubiquitin ligase components CUL-6, SKR-3, 4, 5 and RCS-1. PALS-22 and PALS-25 are two members of the broadly expanded family of 39 PALS proteins encoded in the *C. elegans* genome, but share no detectable sequence similarity with other proteins of known structure or function. Preliminary research shows that PALS-22 and PALS-25 interact to form a complex, suggesting that PALS-22/PALS-25 physical association may balance these opposing regulators of the IPR and serve as an ON/OFF switch to regulate expression. Moreover, mutations in the cullin ligase complex components-CUL-6 and SKR3, 4, 5-are required for protection from proteasomal stress, indicating that CUL-6-SKR is potentially part of an effector complex of PALS-22/25. However, the structures of PALS-22/PALS-25 and how the switch pair regulates the cullin ligase complex are unknown. We aim to express PALS-22, PALS-25 and the CUL-6-SKR-3 ligase complex, and investigate the molecular mechanism of IPR pathway by solving the structure of these proteins using X-ray crystallography. Bioinformatics analysis of the PALS-22 and PALS-25 protein sequences suggest that both proteins may contain helical bundles such as coiled-coils. Recently, we have successfully purified PALS-22 and are working to express soluble PALS-25, possibly by co-expressing PALS-25 with its binding partner PALS-22. In parallel, we are working to co-express the components of the cullin ligase complex-SKR-3 and CUL-6. Our biochemical and structural studies of the interactions of PALS-22/PALS-25 and the cullin-RING ubiquitin ligase complex will give the insight into how these key factors contribute to the IPR pathway in *C. elegans*.

P747/B765

Single-molecule an alysis of the Role of Fad in the Conformational Landscape Od Drosophila Cryptochrome.

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The circadian clock regulates biological and behavioral adaptations in response to day-night cycles. Cryptochromes (CRYs) are members of a family of photolyases involved in DNA repair that are responsible for the oscillation of the circadian clock. CRYs harbor a conserved flavin adenine dinulcleotide (FAD), which is reduced by light and induces conformational changes to activate the

protein. The underlying mechanisms of the light-induced conformational changes of CRYs coupled to FAD reduction, however, are still not well understood. In this study, we use optical tweezers to study the conformational landscape of the *Drosophila* CRY (dCRY) in the presence and absence of FAD, and in light and dark conditions. In the presence of FAD, dCRY unfolds in several steps whose total extension correspondS to the fully folded protein. In contrast, in the absence of FAD the protein fails to fold into its native tertiary structure. By titrating FAD and monitoring the fraction of folded protein, we constructed a single-molecule binding curve and determine a Kd for FAD of 2 nM. Interestingly, the flavin mononucleotide (FMN) moiety binds with a Kd similar to that of FAD and promotes complete folding, whereas the dinucleotide moiety (adenine and phosphate groups) contribute very little to the binding interactions of FAD and has no effect on the fold of the protein. These results indicate that FMN is the dominant moiety responsible for the binding energy of FAD, and that FAD is strictly required for the global structural integrity and stability of the protein. Lastly, the individual unfolding steps in the dark and light conditions indicate slightly higher unfolding forces in the dark state. This result may explain the kinetics of proteasome degradation of dCRY: the light-activated dCRY is less stable which is favored for the cell energetically to remove the protein at the end of each day cycle in response to the environmental lights.

P748/B766

Molecular Mechanism Underlying Protein and Ribonucleic Acid Aggregation.

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For a decade, protein aggregation has been considered to be a main cause of several diseases including Alzheimer's disease (AD) and Parkinson's disease (PD). Many researchers have been struggling to understand the molecular mechanism underlying protein aggregation. Up to now, one of the leading theories is that Mg^{2+} and Ca^{2+} are the two main metal ions that function to create protein aggregates in cells. That being said, there is a very low concentration of metal ions in cells, present only on a micromolar scale. In fact, many millimolar of metal ions are required to induce such protein aggregation. Here, we are interested in finding very small molecules on a millimolar scale that are multivalent, since aggregated proteins are hyper-phosphorylated. Among small molecules in cells, we were interested in the two polyamines spermine and spermidine since they are highly positively charged and exist in high concentration in human cells. Nucleoside triphosphates (NTPs) has three phosphorylated groups that interact electrostatically with polyamines, making them a good model to explain the creation of RNA granules as well as the aggregation of phosphorylated proteins. In this study, we investigated spectral changes of NTPs as a function of polyamine concentration. We found that the absorption band of ATP red-shifted and that of GTP showed a different spectral feature: π-π^* transition red-shifted and *n*-π^* transition blue-shifted with increasing concentration of polyamines. In addition, we found that a new absorption band of ATP and GTP appeared and evolved over time. This indicates that the aggregates of ATP and GTP grow over time. Thus, we conclude that polyamines are very critical in creating biomolecular aggregates.

P749/B767

Studying Habituation in Single-cell Ciliate *Stentor Coeruleus.*

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Habituation, the decrease in an organism's reaction to repetitive stimulations, is studied extensively in metazoans. Intriguingly, a number of single-cell organisms have been shown to exhibit habituation as well. *Stentor coeruleus* is a particularly good model system to study habituation in single cells. *S. coeruleus* response to mechanical stimulations is very clear-cut (the cell contracts from a trumpet-like shape to a sphere), which simplifies the interpretation of the results, and the time scale of the experiment is rapid. Its large size (about 1 mm long) and vibrant color simplify the imaging and image analysis. We developed Arduino-based setup that allows to mechanically stimulate the cells (by shaking their container) in a fully automated fashion. The reaction of the cells to a series of stimulations was imaged with a microscope, and the images processed to extract the dynamics of *Stentor* habituation. The advantages of this method include its fully automated nature and the ability to control the timing of the stimulations via the Arduino, which gives a lot of flexibility in experimental design, as well as allows to track the response of individual cells throughout the habituation experiment. We have shown that *Stentor* habituation can be modeled using a two-state model. Using this model, we have explored a range of habituation parameters both experimentally and computationally.

P750/B768

Utilizing Scanning Probe Microscopy for Biophysical Characterization of Fission Yeast.

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Cells continuously contribute to and remodel their extracellular environment, which in turn helps them to maintain their structure and interact with their surroundings. The fission yeast, *Schizosaccharomyces pombe*, generates a proteoglycan cell wall that serves as a protective barrier, structural support, and resistive force to cellular turgor pressure. Despite having a detailed understanding of its composition, only a limited number of studies have explored biophysical properties of the *S. pombe* cell wall. Atomic Force Microscopy (AFM) is a form of scanning probe nano-microscopy that uses a cantilever to trace a biosurface, obtaining a topographical scan and/or applying force to measure biophysical properties such as surface stiffness, adhesion, and roughness. In this study, we utilized AFM for the first time in quantification of fission yeast cell wall elasticity to better understand how it contributes to overall cellular structure and function. We also evaluated cell wall stiffness in response to osmotic stress and observed increased elasticity. These initial measurements provide a baseline for investigating how fission yeast cell wall stiffness varies under different environmental pressures and how individual cell wall components contribute to the overall strength and stability.

P751/B769

Measuring Protein Concentrations in Biomolecular Condensates Via Quantitative Phase Microscopy.

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Many membrane-less compartments in eukaryotic cells are protein-rich biomolecular condensates formed via macromolecular phase separation from the cyto- or nucleoplasm. The resultant coexisting phases are characterized by differences in composition. Condensates are typically highly enriched in a relatively small number of distinct “scaffold” protein and RNA species relative to the coexisting dilute phase, with additional modest enrichment in a much larger set of “client” molecules. Although it is clear that knowledge of condensate composition is essential for a full biophysical description of the physicochemical properties and associated functions of biomolecular condensates, measurements of composition provide a number of technical challenges, even in minimal model systems of a few purified components. To address this, we use quantitative phase microscopy and optical diffraction tomography to measure the 3D refractive index distribution of model condensates formed in vitro. Here, model condensates are formed by phase separation of purified protein constructs derived from the primarily disordered RNA-binding domain (RBD) of TAF15, a member of the well-studied FUS/EWSR1/TAF15 protein family. From the measured refractive index, we calculate the protein concentration of the condensed branch of the two-phase coexistence curve. Surprisingly, we find that phase separation of TAF15(RBD) is only weakly attenuated by salt, with robust phase separation observed at KCl concentrations ranging from 50 mM to more than 3 M. This suggests that, despite the large number of charged residues in the RBD, coulomb interactions play only a minor role in the protein-protein interaction potential. We find that the protein concentration in TAF15(RBD) condensates is a decreasing function of temperature, indicative of the existence of an upper critical solution temperature beyond 50 C. Interestingly, we also find that partition coefficients determined by confocal fluorescence microscopy dramatically underestimate the concentrations of scaffold proteins in condensates. A simple mathematical model incorporating inner filter and ground-state depletion effects suggests that the discrepancy stems primarily from differences in the fluorescence quantum yield between the dilute and condensed phases.

P752/B770

Impact of the Ecm Stiffness and Geometry in the Collective Dynamics of Epithelial and Mesenchymal Lung Cells.

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The cell microenvironment is composed of a wide range of mechanical stimuli that influence the behavior, fate and function of cells. Some originate from the local intrinsic properties of the materials that constitute the extracellular matrix, such as: stiffness, geometry and chemical composition. In addition, cell microenvironment is in constant dynamic activity since it can be modified by the cells. These stimuli in the right values maintain the cell homeostasis and any disturbance of these parameters may induce a response of cells. Due to alterations in collective behavior are associated with pathological processes such as cancer metastasis and fibrosis, it is necessary to study and characterization topological and mechanical intrinsic properties of cell microenvironment, as well as how those properties influence

the collective dynamics (long range directional order, self-organization) of cells that form organs and tissues. In order to elucidate the impact of the stiffness and geometry of the extracellular matrix on cells collective dynamics first, we seeded alveolar epithelial cells (A549) on polyacrylamide hydrogel substrates with different stiffnesses. Besides, the system was disturbed by adding the transforming growth factor beta (TGFβ) to the A549 cell culture, since TGFβ is related to the mesenchymal epithelial transition and generally modifies the cell morphology. Therefore, the morphological and phenotype of the cells were analyzed by immunofluorescence and western blot assays, which were compared with normal human lung fibroblast (NHLF). Then, we approached the problem of collective dynamics by modelling the epithelium and fibroblasts as an active nematic liquid crystal that presents a long range directional order. We observed a decrease of the activity in the cells seeded on stiffer hydrogels together with the emergence of topological defects, whereas in soft gels the activity increased, interfering with the collective nematic order. Finally, cells were seeded on alveoli-shaped structures of polyacrylamide hydrogels in order to study the influence of geometric constraints on the collective dynamics and cells phenotype. Furthermore, we also observed an heterogeneous mechanical response of the cell layer associated to the stiffness and geometric constraints of the substrates. These results provide a deeper insight towards the understanding of the cellular response to the combined influence of several external stimuli in different pulmonary pathologies associated with an increase in lung tissue stiffness and overexpression of TGFβ.

Development and Morphogenesis: Neural/Eye Development

P753/B772

Pou3f4 in the Otic Mesenchyme Is Essential for Development of Normal Spiral Ganglion Neuron Innervation Patterns in the Mammalian Cochlea.

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Mutations in the Pitt-Oct-Unc (POU)-domain transcription factor *Pou3f4*, located on the X-chromosome are associated with X-linked nonsyndromic hearing loss in the DFNX2 locus. Pou3f4, which is specifically expressed by otic mesenchyme cells, regulates the expression of various Ephs and Ephrin proteins that act as axon guidance cues for spiral ganglion neurons (SGNs). During development, SGNs interact with otic mesenchyme cells prior to forming synapses with hair cells. I will discuss our preliminary findings on how Pou3f4 normally inhibits *Efna1* (Ephrin-A1) and *Efna2* (Ephrin-A2) ligand expression. RNAScope assays show *Efna1* and *Efna2* mRNAs are normally expressed at low levels by otic mesenchyme cells, and that their expression increases about two-fold in *Pou3f4* knockout cochleae. These studies were carried out using mice at E15, which is the time when SGNs begin to fasciculate as they innervate hair cells. Preliminary chromatin immunoprecipitation assays have identified binding sites for Pou3f4 in Efna1 and Efna2 genes. Initial analyses of Efna1 and Efna2 double knockout cochleae show subtle differences in SGN innervation patterns compared to WT littermate controls. Both Ephrin-A1 and Ephrin-A2 attract growing SGN processes in in vitro assays. Hence, we hypothesize that Ephrin-A1 and Ephrin-A2 proteins on otic mesenchyme cells serve as attractive cues for SGN axons and are normally repressed by Pou3f4 to promote axon fasciculation. Additionally, I will discuss our ideas for future work to determine the Eph receptor binding partners for Ephrins-A1 and -A2 on SGNs and genetic experiments to determine whether *Efna1* and *Efna2* knockout alleles can rescue the SGN fasciculation defects observed in *Pou3f4* knockout cochlea. Determining the molecular basis for proper cochlear innervation

patterns through these studies and others will help in developing therapeutics for people with sensorineural hearing loss.

P754/B773

Development of Ependymal and Postnatal Neural Stem Cells and Their Origin from a Common Embryonic Progenitor.

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The adult mouse brain contains an extensive neurogenic niche in the lateral walls of the lateral ventricles. This epithelium, which has a unique pinwheel organization, contains multiciliated ependymal (E1) cells and neural stem cells (B1). This postnatal germinal epithelium develops from the embryonic ventricular zone, but the lineage relationship between E1 and B1 cells remains unknown. Distinct subpopulations of radial glia (RG) cells in late embryonic and early postnatal development either expand their apical domain >11-fold to form E1 cells or retain small apical domains that coalesce into the centers of pinwheels to form B1 cells. Using independent methods of lineage tracing, we show that individual RG cells can give rise to clones containing E1 and B1 cells. This study reveals key developmental steps in the formation of the postnatal germinal niche and the shared cellular origin of E1 and B1 cells.

P755/B774

Scribble and Vangl2 Interact to Influence Neural Tube Morphogenesis.

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Neural tube defects (NTD) occur in 1:500 pregnancies resulting in significant health effects or even death for affected infants. Neural tube closure in vertebrates relies on convergent extension, driven by polarized cell intercalation, and bending of the epithelium through apical constriction. These processes are regulated by the Planar Cell Polarity (PCP) pathway and mutations of PCP proteins such as Vangl2 result in NTD. In addition, mutation of the gene encoding Scribble (Scrib) which is best known for its role in regulating apical-basal polarity and junctional organization, also causes NTD. *Line90 Scrib mutants* (*Scrib^{L90}*), containing a point mutation in the N-terminal LRR domain, display open neural tube (ONT). Interestingly, *Scrib^{L90} mutants* genetically interact with *Vangl2 Loop-tail mutants (Vangl2^{Lp})* to cause NTD. However the molecular mechanisms by which Scrib and Vangl2 interact, and the cellular and biochemical effects of *Scrib* mutations on neural tube development remain unknown. **We hypothesize that Scrib and Vangl2 cooperatively interact to promote polarized cell intercalation and apical constriction during neural tube morphogenesis. To** assess interactions between Scrib and Vangl2 we compared the neural phenotypes of double heterozygotes resulting from *Scrib^{L90} mutants* crossed to either *Vangl2^{Lp} mutants* or mutants with knockout of *Vangl2* (*Vangl2^{KO}*). While 80% of *Scrib$^{L90-/-}$* exhibit ONT only 16% of *Scrib$^{L90+/-}$ Vangl2$^{Lp+/-}$ and* 14% of *Scrib$^{L90+/-}$ Vangl2$^{KO+/-}$* do so. Additionally, our preliminary data show that *Scrib$^{L90-/-}$ Vangl2$^{KO-/-}$* do not exhibit more severe phenotypes than either *Scrib$^{L90-/-}$* or *Vangl2$^{KO-/-}$ mutants* alone suggesting Scrib and Vangl2 act within the same pathway to influence neural morphology. Interestingly mature *Scrib$^{L90+/-}$ Vangl2$^{Lp+/-}$* females all present with imperforate vagina, suggesting the interaction between Scrib and Vangl2 also influences the development of the female reproductive tract. This result along with the observation that *Scrib$^{L90+/-}$ Vangl2$^{KO+/-}$* phenotypes are less

severe than *Scrib*$^{L90+/-}$ *Vangl2*$^{Lp+/-}$ suggest that the *Vangl2*$^{Lp+/-}$ mutation acts in a dominant negative manner to affect Scrib function. Though it is clear that the interaction between Scrib and Vangl2 disrupts neural tube formation, how the loss of these proteins affects neural cell behavior remains unknown. Our preliminary live imaging data show that *Scrib*$^{L90-/-}$ *mutants* do not undergo proper convergent extension. Future studies will detail the effects of loss of Scrib on polarized cell intercalation and apical constriction through live imaging analysis of neural cell behavior. These studies will determine the cellular and molecular mechanisms by which Scrib regulates tissue structure and organization during mouse neural tube development.

P756/B775

The Chemokine-like Protein Fam19a3 Is Expressed by Cone Photoreceptors and OFF Cone Bipolar Cells in Mouse Retina.

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Chemokine signaling has been implicated in retinal development, homeostasis and diseases. Secretion of chemokines by retinal pigment epithelium, Muller cells, astrocytes and microglia has been extensively studied in pathologies. However, little is known about the constitutive expression of chemokines by healthy retinal neurons and their function in retinal development and function. Transcriptome profiling of flow-sorted GFP+ cells in *Nrl*-GFP:*Nrl*$^{-/-}$ (S-cone-like cells) and *Nrl*-GFP:*Nrl*$^{+/+}$ (rod photoreceptors) at postnatal day (P)2, P4, P6, P10, P14 and P28 demonstrated that Fam19a3, a brain-specific chemokine-like protein is highly expressed in S-cone-like cells suggesting a specific role of this gene in cone function. We further analyzed the expression of Fam19a genes (*Fam19a1-5*) in our transcriptome data and found that *Fam19a3* was the only member of the family enriched in S-cone-like photoreceptors. Using in *situ* hybridization, we explored the expression of Fam19a3 during normal retina development and found that *Fam19a3* was specifically expressed by cones and bipolar cells. Fam19a3 protein expression was analyzed using a *Fam19a3-FLAG-T2A-Cherry* reporter mouse model and the protein was detected in cone photoreceptor inner segment and cell body as well as in type 3b OFF cone bipolar cells. Moreover, we observed a gradient in Fam19a3 expression during development that recapitulates bipolar cells maturation. Our studies demonstrated that *Fam19a3* is specifically expressed in cone photoreceptors and OFF cone bipolar cells in retina. The specific expression pattern of Fam19a3 suggests a role of this protein in the development, function and/or maturation of these cell types.

P757/B776

An Adhesion Code Enables Robust Pattern Formation in the Zebrafish Spinal Cord.

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An outstanding question in embryonic development is how different cell types reach their final positions correctly, despite large scale cellular re-arrangement during tissue morphogenesis. To achieve this, cell fate specification needs to be coordinated with regulated cell migration and adhesion, yet the mechanisms are not well-understood. In the zebrafish spinal cord, thirteen neural progenitor domains, each consisting of distinct cell type, are specified along the ventral-to-dorsal axis by opposing gradients of Shh and BMP/Wnt. The Shh signal is noisy, resulting in cells specified in a mixed pattern, requiring that individual cells be sorted to resolve this mixed pattern into well-separated domains. Here we set out to test if differential adhesion plays a role in assisting pattern formation during zebrafish spinal cord

development. We developed two cell-based mechanical assays based on micropipette aspiration to measure adhesion forces and adhesion preferences among three types of neural progenitors (p3, pMN, and p0 cells). Interestingly, each cell type exhibited preference to selectively stabilize homotypic contact and adhered more strongly to cells of the same type. A subsequent transcriptomic and genetic analysis revealed three adhesion molecules (N-cadherin, Cadherin 11, and Protocadherin 19) that are differentially expressed among the three cell types, forming a three-molecule adhesion code. When the expression levels of these adhesion molecules are perturbed, the adhesion preference to cells of the same type is lost, and the neural progenitor pattern in the spinal cord is disrupted in *vivo*. These findings allow us to propose an "adhesion code" mechanism that promotes cell sorting during tissue scale patterning. While the conventional view of spinal cord patterning is heavily focusing on interpretation of biochemical signals and transcriptional regulation, our findings suggest cell adhesion is a critical contributor to enable precise patterning in a tissue that is undergoing dramatic morphogenetic movement.

P759/B778

Signaling and Mechanics-based Models of Posterior Lateral Line Self-organization.

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The posterior Lateral Line primordium is a group of about a hundred cells that migrates under the skin from near the ear to the tip of the tail periodically forming and depositing neuromasts to pioneer formation of the zebrafish Lateral Line sensory system. We describe how local activation coupled with long-range inhibition, operating via both signaling and mechanical interactions, potentially contribute to periodic formation and deposition of neuromasts by the migrating primordium. Wnt and Fgf signaling locally inhibit each other in leading and trailing zones of the migrating primordium, respectively, while Fgfs secreted by leading cells activate their receptors at a distance to promote Fgf signaling in a trailing zone. We use agent-based modelling to show how these interactions can determine periodic formation of Fgf signaling centers in the wake of a progressively shrinking, initially broad, Wnt domain. Center-biased Fgf signaling centers generated by these interactions provide the context for specification of central cells as sensory hair cell progenitors via Notch mediated lateral inhibition and the periodic reorganization of cells to form epithelial rosettes in the developing neuromasts. Independent observations suggest that primordium cells form adhesive links with cells within a defined neighborhood and pull them closer. As cells get closer, more cells can be pulled in. This local aggregation is inhibited by traction forces associated with collective migration, and the balance influences the emergent number and size of self-organized epithelial rosettes. We show how agent-based models based on such mechanical interactions can also make effective predictions about the pattern of neuromast formation and deposition by the migrating primordium under a variety of experimental conditions.

P760/B779

Defective Axonal Transport of Presynaptic Cargoes Associates with Autism-like Synaptic Dysfunction and Social Behavioral Traits.

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The formation of new synapses and maintenance and remodeling of mature synapses require effective axonal delivery of newly synthesized presynaptic cargoes from the soma to distal synapses, raising the

fundamental question of whether impaired axonal transport is associated with neurodevelopmental disorders such as autism spectrum disorders (ASDs). ASDs are characterized by impaired social interactions and communication along with restricted interests and stereotyped repetitive behaviors. While postsynaptic mechanisms play an important role in susceptibility to ASD, a direct link between altered axonal transport and autism etiology has not been established. Specifically, it remains unknown whether altered axonal transport of presynaptic cargos and thus reduced formation, maturation, or maintenance of presynapses contribute to ASD-like synaptic dysfunction and behavioral abnormalities. Investigations into axonal transport mechanisms in an in *vivo* model system will provide the essential information necessary to identify core presynaptic defects of ASDs. Here, we report that altered axonal transport of presynaptic cargos and thus reduced formation and maturation of synapses are one of core presynaptic mechanisms underlying synaptic dysfunction and social behavioral abnormalities. Syntabulin acting as a development-regulated motor adaptor linking kinesin-1 and presynaptic transport cargos. Syntabulin expression in mouse brain peaks during the first two weeks of postnatal development and progressively declines during brain maturation. Conditional *syntabulin*$^{-/-}$ mice (*stb* cKO) display impaired axonal transport of presynaptic cargos, reduced synapse density and active zones, and altered synaptic transmission and long-term plasticity. Intriguingly, *stb* cKO mice exhibit core autism-like traits, including defective social recognition and communication, increased stereotypic behavior, and impaired spatial learning and memory. Our study is further confirmed by an autism-associated missense STB variant that loses its motor adaptor capacity and fails to rescue *stb* cKO phenotypes. These disrupted synaptic plasticity and abnormal behaviors establish the *stb* mutant mouse as a promising autism model for investigations into the core presynaptic defects at the onset of ASDs. Thus, our study elucidates a new axonal transport mechanism underlying synaptic and behavioral abnormalities that bear similarities to human autism (Supported by the Intramural Program of NINDS, NIH).

P761/B780

Identifying New Developmental Functions for Genes Associated with Intellectual Disabilities.

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Intellectual disabilities (ID) are complex neurodevelopmental disorders that affect a significant portion of the general population and can be an immense health issue. Genetic mutations caused by rare copy number variants (CNVs) have been shown to play a role in ID and severe developmental delays. In particular, one CNV was identified in a subset of children diagnosed with ID that results in a heterozygous deletion of six genes at chromosome 16p12.1. Individuals with this deletion can display a variety of additional symptoms including craniofacial abnormalities, microcephaly, cardiac defects, seizures, and growth retardation. Some patients also have a second CNV mutation, or 'second-hit', elsewhere in the genome, which increases the severity of their symptoms. While some information is known regarding the function of the genes associated with this deletion, it is still unclear what their roles are in the context of vertebrate embryonic development. Thus, we sought to elucidate the function of these genes during development using genetic manipulation strategies to reduce gene dosage and observe phenotypic differences in *Xenopus laevis.* We have found that reduction of three genes, *MOSMO*, *POLR3E*, and *UQCRC2* cause severe craniofacial and cartilage defects. Additionally, we have found that reduction of *MOSMO* decreases axon length, and knockdown of *POLR3E and MOSMO* cause severe brain defects. Moreover, simultaneous knockdown of *MOSMO* with either *SETD5* or

POLR3E enhances several developmental phenotypes. Together, our results suggest that some genes within the 16p12.1 region are essential for proper embryonic development, providing further insight into how each of these genes may be contributing to the phenotypes associated with the 16p12.1 deletion.

P762/B781

Depletion of *Pxdn* in Mice Leads to Severely Disorganized Eye Structure in Homozygote in Contrast to Normal Visual Function in Heterozygote.

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Peroxidasin (PXDN) is a unique peroxidase containing extracellular matrix motifs, and stabilizes collagen IV network by creating covalent sulfilimine crosslinks. PXDN gene knockout in *C. elegans* and *Drosophila* results in demise at embryonic and larval stages. PXDN mutations lead to severe eye disorders including microphthalmia, cataract, glaucoma and anterior segment dysgenesis in human and mice. To investigate whether PXDN deficiency affects morphological phenotype and organ development in mice, we generated *Pxdn* knockout mice by deletion of a sequence containing 5'UTR and exon1 of *Pxdn* gene using CRISPR/Cas9 system. Loss of both PXDN expression and collagen IV sulfilimine cross-links was detected only in the homozygous mice, but there were no changes detected between the heterozygous and wild type mice. Only the homozygous mice also showed completely closed eyelids or almost closed eyelids with small eyes, with no apparent external morphological defects in brain, heart, lung, spleen, kidney, and testis. In the histological analysis of eye tissues, the homozygous mice had extreme defects in eye development by showing no eyeballs or drastically disorganized eye structures, whereas the heterozygous mice showed normal eye structure. Visual function tests also revealed no obvious functional abnormalities in the eyes of the heterozygous mice compared with those of wild type mice. Thus, these results point to the essentiality of PXDN in eye development, and also indicate that single allele of *Pxdn* gene is sufficient for eye structure formation and normal visual function.

P763/B782

An Ectodermal Stem Cell Niche Drives Two-step Regeneration of Suicide Cells.

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Nematocytes, the "stinging" cells found in cnidarians (corals, jellyfish, etc), are defined by two important features: their novel organelle (the harpoon-containing nemato*cyst*) and their capacity for regeneration/replacement. Upon discharge, the contents of the nematocyst (harpoon, venom, etc) are forcefully expelled with nanosecond speed, tearing open the apical cell membrane, and necessitating cellular regeneration; thus, nematocytes have been termed single-use or "suicide" cells. We capitalized on this capacity for replacement to investigate the molecular regulation of organelle synthesis in this novel cell type. Nematocytes were discharged from the tentacle tips of the model sea anemone,

Nematostella vectensis, using an XYClone infrared laser ablation system. We show that complete replacement of the tentacle tip nematocytes occurs in under 24h and this process does not require cell proliferation. Using a specific marker of nematocyst synthesis, we show that wildtype animals regenerate nematocytes from a population of stem cells in the tentacle ectoderm. Initial synthesis of the post-golgi vacuole and proper trafficking of cnidocyte-specific proteins occurs during the first 24h after division from the nematocyte progenitor, giving rise to a population of partially differentiated nematocytes in the base of the tentacle tip. Final organellar maturation is cued upon discharge of the distal population of mature nematocytes. Treatment with hydroxyurea (HU), a reversible inhibitor of cell proliferation, blocked division of new nematocytes from their progenitors but had no effect on organellar maturation of the partially differentiated population. Treatment with DAPT, a gamma-secretase inhibitor which interferes with Notch signaling, inhibited final maturation but had no effect on the early steps of organelle synthesis. These results demonstrate a two-step process underlying nematocyte replacement and definitively identify, for the first time, a resident population of ectodermal stem cells in the tentacle of *N. vectensis* as the source of new nematocytes.

P764/B783

Transcriptome an alysis of Plasticity in the an terior-Posterior Neural Axis in *Xenopus Laevis* Embryos.

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While plasticity is an integral component of all living tissues, embryos display a profound degree of robustness. To analyze the limits of this robustness, we performed several different types of perturbations on the anterior-posterior neural axis of Xenopus laevis embryos. First, presumptive neural tissue was removed from a labeled donor, rotated 180° and heterologously transplanted into an unlabeled host embryo. Second, as a control for the rotation effects, tissue was removed from a labeled donor and heterologously transplanted into an unlabeled host embryo unrotated. Finally, as the sham may be slightly out of register when placed into a host embryo, an autologous (“selfie”) transplant was performed where the tissue was removed and then replaced back into the same embryo. All experiments were performed at both mid- and late gastrula stages, and raised to neurula or hatching stages. An initial series of experiments employed neural regional markers using in-situ hybridization as a molecular readout and analyzed in histological sections. All transplants healed and incorporated neighboring tissue. Moreover, all embryos showed profound patterning regulation except those with an inversion of their axis at the late gastrula stage. In order to determine the molecular basis of early developmental plasticity, we conducted a global gene expression RNA-Seq experiment on all embryo transplants and control. Our RNA-Seq data suggest a model in which aspects of early neural plasticity are associated with unique signatures of pathways and their associated genes. One set of pathways is enriched in all classes of transplants that heal and may mediate the ability of any type of transplant to incorporate and heal into neighboring tissue. This entails the upregulation of ubiquitination apoptosis and oxidative stress genes. Our transcriptome analysis reveals a second, distinct combination of pathways and genes that incorporate and heal, but also appropriately re-pattern the anterior-posterior axis. Maintaining plasticity in these transplants was correlated with expression of neuronal transcription factors, chromatin modifiers, Wnt signaling, calcium signaling, and pattern recognition molecules. In addition to identifying gene pathways involved in plasticity, the complexity and number of comparisons has necessitated a novel Baysesian approach to analysis of the RNA-Seq data.

P765/B784

Impaired *De Novo* Notochord Vacuole Formation Leads to Chordoma.

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The notochord is a conserved axial component that serves as a structural scaffold. It is required for embryonic axis elongation and proper spine development. In zebrafish, it consists of a core of giant vacuolated cells surrounded by an epithelial-like sheath. Previous work showed that the fluid filled vacuole present in each vacuolated cell is a lysosome related organelle (LRO) that forms via a specific post-Golgi biosynthetic trafficking pathway and that vacuole integrity is important for notochord function. However, the machinery that controls *de novo* vacuole formation and integrity is not known. The sheath cells that surround the notochord are also capable of acquiring a vacuole and can restore notochord integrity upon mechanical damage. Once notochordal cells acquire a vacuole they become post-mitotic. In humans, notochordal cells that continue to divide outside of the intervertebral disc form chordomas, a poorly understood and aggressive type of tumor. Here, we have used a two-pronged transcriptomics and proteomics approach to identify proteins involved in *de novo* vacuole formation. We find that loss of a protein previously linked to LRO function leads to fragmentation of notochord vacuoles and impaired axis elongation. Interestingly, upon injury of the notochord, sheath cells fail to form a fully inflated vacuole and continue to grow outside of notochord boundaries, forming a tumor-like mass. This work identifies a protein important for notochord vacuole biogenesis in zebrafish and provides a new model to investigate chordoma formation.

P766/B785

Role of Neurofibromin 2 in Optic Fissure Closure and Retinal Pigment Epithelium Regeneration.

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Purpose: Neurofibromin 2 (NF2), an activator of the Hippo pathway, regulates retinal pigment epithelium (RPE) cell fate and proliferation during eye development. One important process in development is optic fissure (OF) closure, as failure to properly close the OF is known to lead to coloboma. Furthermore, once the eye is fully developed, many cells types including the RPE, lose their potential to proliferate and regenerate. As the Hippo pathway is known to regulate development and cell proliferation, we focus on manipulating the Hippo pathway through NF2 in the adult RPE. Our study has two main goals: investigate the role of NF2 (1) during OF closure and occurrence of coloboma and (2) during RPE regeneration. **Methods:** We generated NF2 conditional knockout (CKO) embryos using the constitutive ocular *Rx3*-Cre*. Nf2*-CKO embryos were analyzed during OF closure for changes in cell proliferation, apoptosis, tissue patterning, apicobasal polarity, and cellular junctions. To study RPE regeneration, we generated *Nf2*-CKO mice using the RPE-specific, inducible tet-on *VMD2*-Cre*.* Mice were injected with sodium iodate, an injury model that mimics the course of age-related macular degeneration. Proliferation and regeneration were assessed by EdU incorporation and expression of OTX2 and RPE65 in the presumptive RPE layer 7.5 weeks post damage. **Results***: as* previously shown, *Nf2-CKO* embryos exhibit a coloboma with a narrow gap at E13.5, indicating a role for NF2 at late stages of OF closure. Early during OF closure, we observe a significant increase in EdU-labeled RPE cells, particularly in the ventral eye. Additionally, cell polarity and apoptosis appear largely maintained in the OF margins. Later during OF closure, the cell number in the ventral RPE is increased in *Nf2*-CKOs, and RPE markers persist in the OF margins. Currently, we are attempting to rescue OF closure. In adult

damaged RPE, our preliminary results show that there is a tendency for increased proliferation in *Nf2*-CKOs. Furthermore, there are more OTX2-positive cells in the presumptive RPE, and several show colocalization with EdU. This suggests potential de-novo production of RPE cells. Currently, we are analyzing markers for Hippo pathway components, cell junctions, and proliferation. **Conclusions:** Our results indicate a role for NF2 during OF closure, possibly by restricting the proliferation of RPE lining the OF margins. Our results also indicate a potential role for Hippo pathway modulation in regenerative proliferation of mature RPE. We hypothesize that NF2 acts through regulation of the Hippo/YAP-TAZ pathway during development and regeneration, consistent with previous studies showing a role for YAP-TAZ in regulating RPE fate.

P767/B786

Role of Calcium Activity during Early Neural Development.

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Influxes of calcium ions (Ca^{2+}) that occur at different amplitudes, frequencies, and cellular locations regulate a wide array of physiological processes. While calcium activity is indispensable for all aspects of embryonic development, particularly the developing nervous system, its dynamics during early embryonic development remain poorly understood. For instance, it is not known if there is a conserved or stereotypical pattern of calcium activity among embryos at a given stage of development of a given species. This is due to apparent irregular patterns of activity of individual cells, and a general lack of comprehensive analysis techniques at cellular level in *vivo*, where cells are constantly in a state of division, cell-cell intercalation, and migration. In this study, we aim to address this need and understand the spatial-temporal pattern of Ca^{2+} activity at a cellular level during vertebrate neural development. To this end, we devised a method to analyze the spatial-temporal pattern of calcium activity by constructing a composite neural plate. First, we monitored calcium activity of the *Xenopus laevis* neural plate using the genetically encoded calcium marker GCaMP6 and constructed a composite neural plate registering images onto a cellular-resolution grid. Secondly, the activity of individual cells was quantified using multiple analysis methods, including spike counting and entropy, and subsequently visualized as heatmaps in the composite neural plate. Our results, consistent with previous reports, show that neural plate stage embryos exhibit elevated calcium activity compared to late gastrula stages. In addition, our approach allows us to correlate calcium activity pattern with other cellular attributes, including levels of gene expression, cell size changes, and cell movements. We demonstrated the applicability of our method by correlating spatial-temporal pattern of calcium activity with expression pattern of a pan-neural marker gene, *Sox2*. Our preliminary results suggest calcium activity is significantly correlated with expression of *Sox2 in* specific neural regions. Moreover, our analysis demonstrates that while calcium waves occur in a stereotypical and consistent pattern among embryos at a given stage, spiking activity appears to be stochastic. Our approach of combining information on cellular attributes from different regions of a tissue into a composite template can be useful in facilitating spatial-temporal pattern analysis and therefore elucidating many cellular processes.

Embryogenesis

P768/B787

A Spatial Gradient of Cell Size Controls Genome Activation and Contributes to Vertebrate Early Development.

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Zygotic genome activation (ZGA) is a process in embryonic cells that awakens and begins transcribing thousands of zygotic transcripts. ZGA marks the maternal-to-zygotic transition in which development switches from maternal to zygotic control. ZGA is essential for subsequent gastrulation and germ-layer specification, but how ZGA is triggered in vertebrate embryogenesis is poorly understood. Through metabolically labeling the nascent transcripts and wholemount imaging, our lab has found that in *Xenopus* blastula embryos, genome activation correlates with cells achieving a threshold size, due to blastomere division in the absence of growth. We identified a highly stereotyped spatial and temporal pattern of ZGA that occurs initially in small cells at animal pole and is delayed by two hours in cells of the vegetal pole. This spatiotemporal pattern tightly correlates with the gradient of cell sizes present in the blastula embryo. Although the gradient of cell sizes is evolutionally conserved in lamprey and amphibian species, less is known about its role on regulating the early vertebrate embryogenesis. The goal of our current study is to understand the biological significance of the gradient of cell sizes and the resulting pattern of transcriptional onset. To do so, we have developed an embryo temperature controller to manipulate the cell size gradient. It works by slowing down the division rate of fast dividing cells in the animal pole and speeding up division of slow dividing vegetal cells. By generating *Xenopus* blastula embryos with reversed cell size gradients, we found that the spatial pattern of ZGA was also reversed; genome activation occurred first in vegetal pole and was delayed in animal pole. Additionally, we observed delayed gastrulation and neurulation in embryos with altered cell size gradients. Portions of those embryos show defects on head and gut formation at tadpole stage, perhaps because their animal pole with transcriptional delay miss an important developmental window for responding to inductive signals which are originated in the vegetal pole. This work suggests that cell size gradient is critical to spatial patterns of ZGA and embryonic development.

P769/B788

How Cell Size Controls Genome Activation and Orchestrates Fate Decisions.

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A fundamental question is how embryonic cells induce genome activation and determine their fate in early embryo development. It has been proposed that genome activation is regulated by cell size through a titration mechanism dependent on DNA:cytoplasm ratio. Recently, using a model embryo that contains a gradient of cellular dimensions we demonstrated that the onset of genome activation is dependent on cells reaching a critical threshold size. By physically manipulating the size of embryos, we also demonstrated that reductions in cell size is sufficient to trigger premature genome activation in a dose-dependent fashion. However, it remains unknown what roles of cell size gradients are in early development and whether size thresholds for genome activation are conserved among vertebrate embryos. The origin of the cell size variation in *Xenopus* is due to distinct duration of the cell cycle in

distinct regions of the embryos. Therefore, to investigate the role of the cell size gradient we developed tools to control cell cycle timing in space in the embryo. In doing so, we were able to flatten and even reverse the cell size gradient. Intriguingly, by reversing the cell size gradient we found that the spatial pattern of genome activation was also reversed and that embryo development was also affected. We are investigating how alteration of spatial pattern of genome activation impacts tissue communication and cell fate regulation during gastrulation. Because cell size is thought to derepress transcriptional inhibition through titration of histone proteins we have quantified the spatial distribution of histones in *Xenopus* embryos and characterized a zebrafish mutant embryo that contains reduced levels of the core histones. We found that histone levels explain the size threshold for genome activation as well as cell fate decision. Finally, to address whether a cell size threshold for genome activation is a conserved feature of early embryo development we are imaging the onset of large-scale transcription in space and time in additional model systems, including blastula embryos from *X. tropicalis*, *D. rerio and* others. These studies highlight the functional importance of cell size as a regulatory parameter in genome activation and cell fate control.

P770/B789

Uncoupling Nuclear Size and Cell Size during Early Development: Perinuclear Endoplasmic Reticulum as a Limiting Factor for Nuclear Growth.

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Nuclear size is a particularly well controlled aspect of subcellular morphology which plays a critical role in gene expression, early development and disease. The relationship between cell size and nuclear size established in the early 1900's supported the hypothesis of a nucleus-to-cell size scaling process. However, direct assays to test this scaling and the underlying mechanism were lacking. By monitoring nuclear dynamics in early sea urchin embryos, we found that nuclei undergo substantial growth in each interphase, reaching a maximal size prior to mitosis that declined steadily over the course of development as cell volume decreases. Strikingly, manipulations of cytoplasmic volume through multiple chemical or physical means showed that nuclear size can be uncoupled from cell size, suggesting that cytoplasmic volume is not a limiting factor for nuclear growth in the early embryo. Instead, we observed that the initial pool of perinuclear endoplasmic reticulum is partitioned at each division and progressively reduced. Our data support a new model in which perinuclear endoplasmic reticulum may serve as a limiting local membrane pool that determines subsequent nuclear growth kinetics, establishing size scaling throughout early development.

P771/B790

Blastoderm Formation in Crickets: a Crowding Mechanism Explains Nucleus Speed, Direction, and Division Timing.

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In most insects, development begins as a syncytium: that is, many nuclei divide and move within the single shared cytoplasm of the egg. It is essential for the nuclei to form a single layer—the blastoderm—which becomes the incipient embryo. A longstanding question is how these proliferating and migrating

nuclei self-organize into a single layer with the proper number, timing, and spatial arrangement. Recent work has shed new light on this puzzle in *Drosophila melanogaster*, showing that flies employ cytoplasmic flows and a mechanism that synchronizes divisions. Here we show that the cricket *Gryllus bimaculatus* has an altogether different solution to the problem. By live-imaging yolk and 3D-tracking nuclei in transgenic cricket embryos, we find that: (1) Cytoplasmic flows are unimportant for nucleus movement. (2) Division cycle period varies widely among coexisting nuclei, correlating with local nuclear density. (3) Nucleus speeds and orientations throughout blastoderm formation can be explained by a local crowding mechanism. Based on these findings, we present a simple geometric model that describes embryo-wide patterns of nucleus behavior. We use the model to predict what would happen to nucleus movements if an egg were to be physically constricted. Then we empirically test this prediction by constricting eggs, finding that the resulting nuclear behaviors are consistent with simulated embryos. Finally, we put the cricket and fruit fly data in the greater context of insect diversity. Across insects, syncytial eggs come in an enormous range of shapes and sizes. We hypothesize that over a macroevolutionary timescale, egg size and shape co-vary predictably with aspects of nucleus dynamics in early insect embryos.

P772/B791

Cell Lineage Specific Chiral Actomyosin Flows Drive Cellular Rearrangements in *C. Elegans* Development.

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Cells need to be positioned correctly during embryogenesis for achieving important processes like body axis formation and organ development. The mechanisms by which cells reposition in the early developing embryo are still not completely understood. Our lab recently showed that the gradient of myosin in the actomyosin cortex generates chiral rotatory flows and these flows are important for breaking left-right symmetry in a developing *C. elegans* embryo (Naganathan *et al.*, eLife 2014). Although it has been speculated that the process of spindle elongation play an important role in repositioning cells, we here test the hypothesis that chiral flows at the actomyosin cortex drive cellular repositioning during cell divisions. We show that chiral rotatory flows arise only in the somatic (AB) cell lineage, and that the presence of these flows correlates with cellular repositioning in the embryo. By combining active chiral fluid theory with experimentally measured myosin profiles we deliver a physical model explaining the emergence of chiral flows from myosin gradients during AB-lineage cell divisions. Using reverse genetics approach and temperature sensitive mutants we demonstrate that cellular rearrangements in the AB lineage are driven by chiral actomyosin flows. Thus, we conclude that chiral actomyosin flows drive cellular rearrangement in *C. elegans* early development.

P773/B792

Cell Ratcheting through the Sbf RabGEF Directs Force Balancing and Stepped Apical Constriction during *Drosophila*Gastrulation.

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During *Drosophila* gastrulation, the invagination of the prospective mesoderm is driven by the pulsed constriction of apical epithelial surfaces. Here, we address the mechanisms by which the irreversibility of pulsed events is achieved, while also permitting uniform epithelial behaviors to emerge. We utilize MSD-

based analyses to identify contractile steps and find that when a trafficking pathway initiated by Sbf is disrupted, contractile steps become reversible and AP anisotropies are lost. Sbf localizes to tubular, apical surfaces and associates with Rab35 where it promotes Rab GTP exchange. Interestingly, when *Sbf*/*Rab35* function is compromised, the apical plasma membrane in the ventral furrow become deeply convoluted and nonuniform cell behaviors begin to emerge. Consistent with this, Sbf/Rab35 appear to prefigure and organize the apical surface for efficient and balanced Myosin II network function. Finally, we show that Sbf/Rab35/CME direct plasma membrane to Rab11 endosomes through a dynamic interaction with Rab5 endosomes. These results suggest that periodic ratcheting events shift excess membrane from cell apices into endosomal pathways to permit re-shaping of actomyosin networks and the apical surface.

P774/B793

Post-transcriptional Regulation of Actin Remodeling Genes in the Early Embryo.

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This study investigates the post-transcriptional regulation of actin remodeling genes by microRNA-31 (miR-31), using the sea urchin as a model. Our earlier work indicated that knockdown of miR-31 results in extra spicule rudiment, decreased spicule length, and defective skeletal patterning. In addition, miR-31 suppresses multiple transcription factors and effector genes within the skeletogenic gene regulatory network. To further identify the global function of miR-31, we conducted experiments with biotinylated miR-31 pull down assays and proteomic analyses of control and miR-31 knockdown embryos. Results identified Fascin and Rab35 as additional targets of miR-31. Fascin and Rab35 have been shown to interact with actin to remodel cytoskeletal structures. Results indicate that the spatial and temporal expression of miR-31 is enriched in the perinuclear region in non-dividing blastomeres and seems to associate with mitotic spindles and the chromatin in dividing blastomeres of the early embryo. miR-31 may regulate local translation of its targets to mediate mitosis in early cleavage embryos and regulate cell migration and motility during gastrulation. The results from this study serve as a paradigm for understanding post-transcriptional regulation of actin remodeling genes in ensuring proper cell divisions and cell motility in the early embryo.

P775/B794

Rho Gap RGA-8/ Rich1/SH3BP1 Promotes Myosin Enrichment in Migrating Embryonic Epidermis through CDC-42.

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CDC-42 regulation of non-muscle myosin/NMY-2 is required for polarity establishment in the one cell embryo of *C. elegans*. CDC-42 and NMY-2 continue to regulate polarity throughout embryogenesis, but their contribution to later events of epidermal morphogenesis are less understood. Our previous studies of epidermal enclosure revealed an essential requirement for the GTPase CED-10/Rac1 and its effector, the WAVE/Scar complex, in promoting the protrusions that drive enclosure through activation of the branch actin regulator Arp2/3. Our analysis here of RGA-8, a homolog of SH3BP1/Rich1/ARHGAP17/Nadrin, with BAR and Rho GAP motifs, demonstrates the contribution of CDC-42 and NMY-2 to two events of epidermal morphogenesis: ventral enclosure and elongation. Genetic and molecular data show RGA-8 regulates CDC-42, in a pathway involving the F-BAR proteins

TOCA-1 and TOCA-2, and the CDC-42 effector WSP-1. These proteins affect levels of myosin in the migrating epidermal cells during ventral enclosure, and the timing of enclosure. We propose TOCA proteins and RGA-8 fine-tune CDC-42 activity in the migrating cells, to promote myosin enrichment through effects on the myosin kinase MRCK-1. CDC-42 thus controls myosin during migrations and cell shape changes of epidermal morphogenesis.

P776/B795

Investigation of Wolf-hirschhorn Syndrome Associated Genes in *Xenopus Laevis* Embryos.

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Wolf-Hirschhorn Syndrome (WHS) is a neurodevelopmental disorder characterized by intellectual disabilities, craniofacial abnormalities, seizures, and defects in the development of the skeleton, heart, and urogenital tract. Previous genetic testing has shown that patients with WHS typically exhibit irregularities on the short arm of chromosome 4, such as deletions and microduplications. Most mutations occur in the first critical genomic region, containing the genes WHSC1 and WHSC2. Other WHS-associated genes such as TACC3 and LETM1 are located telomeric to this critical region. We have shown that the protein products of these four genes may be implicated in cranial neural crest cell (CNCC) migration and motility. Neural crest cells are multipotent stem cells found in vertebrates that originate along the neural tube early in development. These cells subsequently undergo a process called epithelial-to-mesenchymal transition (EMT) where they migrate long distances to form structures such as the face, jaw, peripheral nervous system, cartilage, and heart. Using *Xenopus laevis*, we determined that WHS-related genes are expressed in the neural tube and CNCC, leading us to investigate the effect of both single and combinatorial gene knockdown on craniofacial, pharyngeal arch, and cardiac morphology as well as on cartilage formation and CNCC migration. We found that the expression of all four genes is high in migrating neural crest cells and the neural tube, and that when these genes are knocked down, we see a significant defect in the migration of CNCC. We also are investigating how the knockdown of these genes leads to defects in cardiogenesis and specifically cardiac looping. We hypothesize that these defects arise as the direct result of errors in cell migration during cardiac development. This work will help to inform our understanding of the embryonic function of these genes and how their loss contributes to WHS.

P777/B796

Active Transport Allocates Lipid Droplets during Drosophila Embryogenesis, Facilitating Lipolysis and Punctual Development.

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Lipid droplets (LDs) are fat storage organelles linked to numerous human diseases including obesity and diabetes. LDs are highly motile intracellularly. Our understanding of the role of this motility in LD functions is incomplete, but an emergent theme is that trafficking is crucial to proper LD metabolism. During early embryogenesis of multiple animal species, LDs undergo massive intracellular rearrangements. But, while it is known that embryonic LDs play roles in energy production, membrane synthesis, and the generation of signaling molecules, the role(s) of their motion is unclear. It is in *Drosophila* embryos that we have the greatest mechanistic understanding of how LD trafficking occurs. In these embryos, LDs are transported rapidly along microtubules, during the early cleavage and

blastoderm stages. This microtubule-based-transport utilizes both Dynein and Kinesin motor proteins, creating bidirectional transport. Embryos lacking the Klar protein have been previously shown to have severely impaired LD motility, including reduced motor-driven-forces propelling the LDs, massive reduction in the speed of individual LDs, and misallocation of LDs to the yolk cell post cleavage divisions. We find that embryos mutant in a seemingly unrelated LD protein Jabba display a similar misallocation of lipid droplets to the yolk cell, but via a completely different mechanism. Individual LDs in *Jabba* mutant embryos move at wild-type speeds, in stark contrast to *klar* mutants. However, most droplets are present in aggregates with each other or with glycogen granules. These aggregates are poor transport cargoes. Thus, Jabba promotes normal droplet allocation by preventing aggregation, while Klar ensures proper function of the microtubule-motor machinery. Both *klar and Jabba* mutant embryos fail to consume a portion of the misallocated LDs, leaving them with higher triglyceride levels relative to wild-type embryos at hatching. *Klar and Jabba* mutant embryos also take significantly longer to hatch than wild-type embryos, suggesting LD trafficking facilitates consumption and that failed consumption impairs development. Importantly, LD turnover and hatching times are, like LD allocation, due to the maternal functions of Jabba and Klar and do not reflect later, zygotic roles. We have titrated the amount of maternal Jabba protein provided to embryos and found a correlation between Jabba levels and delayed hatching. Preliminary data suggest that in the mutant embryos translation maybe inhibited through 4E-BP1/Thor upregulation, suggesting a cellular underpinning for the developmental stalling. To our knowledge, our data is the first to demonstrate organismal deficits caused by aberrant intracellular LD motility.

P778/B797

Translocation of Pyruvate Dehydrogenase Alpha Is Essential for Zygotic Genome Activation in Porcine Embryos.

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Mammalian zygotic genome activation (ZGA) is critical for embryonic development, which appears after first several cleavages. Nuclear translocation of pyruvate dehydrogenase alpha 1(PDHA1) in mouse embryos during ZGA has been reported. To investigate the function of PDHA1 during ZGA in porcine embryos, a PDHA1 knockout (KO) parthenogenetic embryo model was generated by injection of Cas9 mRNA and sgRNAs. First, the nuclear translocation of PDHA1 gradually enhanced from two to late four-cell stage in porcine embryos. Second, results showed that level of PDHA1 and histone acetylation dropped significantly in PDHA1 KO group. Meanwhile, the 4-cell arrest rate significantly increased at 72 h after activation. Third, the LDH activity decreased and the ATP level increased in the PDHA1 KO group. Forth, the mRNA level of zygotic genes all dropped significantly in the PDHA1 KO group, however, overexpression of nuclear PDHA1 rescued ZGA. Fifth, histone acetylation levels significantly decreased when PDHA1 was targeted, which can be rescued fully or partially by overexpression of PDHA1. In conclusion, the nuclear translocation of PDHA1 promotes histone acetylation for zygotic genome activation in pigs.

P779/B798

Examining the Impact of Mitosis Duration on Embryo Development.

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The timing of cell divisions is highly variable in mammalian embryos, and is a potential indicator of embryo health in fertility clinics (termed morphokinetics). In somatic cells, extended mitosis can cause premature separation of sister chromatids, a phenomenon known as "cohesion fatigue" (CF), which could cause aneuploidy. Separately, a so-called mitotic clock checkpoint (MitClock) has been recently described wherein an extended duration of mitosis can cause a subsequent G1/S arrest. Here, I wish to determine whether cohesion fatigue can occur, and whether the MitClock operates, in mouse preimplantation embryos. We manipulated the duration of mitosis in two-cell stage mice embryos with the anaphase promoting complex inhibitor APCin. Live and fixed confocal imaging of the spindle, kinetochores, and DNA were performed. Mitosis prolongation causes an increase of spindle length and a time-dependent loss of chromosome alignment. 4% of all sister pairs had individualized by 6hours of mitotic arrest, and 66% by 24 hours. The loss of sister cohesion was preceded by an increase in inter-kinetochore distances from 0.59µm to 0.78µm ($p<0.0001$), consistent with CF. 24 hours mitosis prolongation in the presence of a spindle poison did not trigger CF, suggesting it is spindle-tension-dependent. Strikingly, live imaging revealed that 6 hours mitosis prolongation does not prevent subsequent embryo development, but doubled the frequency of micronuclei per embryo (4.5 vs 2.7), suggesting that CF leads to aneuploidy. In contrast, 24 hours mitotic arrest causes a potent cell cycle arrest in the subsequent interphase. Preliminary data suggests that this arrest may be dependent upon a critical level of cohesion fatigue.Moderately prolonged mitoses fail to activate a mitotic clock checkpoint in embryos, but lead to appreciable increase in cohesion fatigue and chromosome segregation defects. Our data allude that cohesion fatigue could be a previously unappreciated cause of mosaic aneuploidy in the mammalian embryo.

P780/B799

Bezafibrate Prevents Aging in in *Vitro*-matured Porcine Oocytes.

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Bezafibrate, a fibrate drug used as a lipid-lowering agent to treat hyperlipidaemia, is a pan-agonist of PPAR. It can prevent mitochondrial dysfunction and antioxidant system disturbance. It was also reported that bezafibrate upregulated PGC-1α and mitochondrial biogenesis. Postovulatory aging induces oxidative stress and mitochondrial damage. But the protective effect of bezafibrate on postovulatory aging in porcine oocytes is little known. In the present study, we investigated the effect of bezafibrate on the ability of against the progression of oocytes aging in *vitro* in porcine as well as the underlying its mechanisms. Different concentrations of bezafibrate (0, 25, 50, 100, 200 µM) was added to IVM medium during in vitro aging (IVA) and IVC medium after activation. Blastocyst formation and its quality were significantly increased in 50 µM bezafibrate addition group. Bezafibrate significantly alleviated IVA induced ROS production and increased GSH levels. However, the mitochondrial DNA copy number was not increased after bezafibrate treatment. Taken together, these results suggest that bezafibrate has beneficial effects against porcine postovulatory oocyte aging by its antioxidant property.

P781/B800

Elucidation of the Function of Mir-31 during Early Development.

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MicroRNAs (miRNAs) are short, non-coding RNAs that repress translation of their target mRNAs. We previously identified that miR-31 is required for development and function of skeletogenic cells of the sea urchin embryo. In order to understand the global function of miR-31 in development, we injected biotinylated miR-31 into sea urchin zygotes to pull down its bound targets. RNA-sequencing was used to identify differentially pulled down transcripts between control and injected embryos in the blastula stage. Additionally, the early blastula proteome from zygotes injected with a scrambled control or miR-31 inhibitor was submitted for mass spectrometry to identify miR-31 targets. Our analysis of both data sets indicated that several potential miR-31 target genes encode proteins that interact with actin, including *Fascin*, *Nr1m3*, and *Rab35*. These results led to our hypothesis that miR-31 suppresses expression of actin remodeling genes to ensure proper formation of embryonic structures. Using a dual luciferase assay and site-directed mutagenesis, we found miR-31 to directly suppress *Fascin* and *Rab35*. Two isoforms of *Fascin* were identified, each with distinct expression patterns. Whole mount in *situ* hybridization revealed that *Fascin a* is expressed in non-skeletogenic mesodermal cells, and is increased during the blastula stage, when these cells are migrating throughout the embryo. In contrast, *Fascin B* is expressed in the ectoderm in the blastula stage, and the ectoderm and endoderm during the gastrula stage. Additionally, *Fascin B, Rab35* and miR-31 are expressed in the midzone between dividing nuclei and enriched perinuclearly in non-dividing blastomeres of cleavage stage embryos. Immunolabeling revealed that Fascin localizes to the cortex of the egg and is ubiquitously expressed with enrichment in skeletogenic cells during the cleavage stage, in the mid-gut, and ciliary band of the larval stage. Preliminary results indicated that inhibition of miR-31 results in Fascin protein enrichment at the perinuclear area of blastomeres of the cleavage stage embryos, consistent with miR-31's perinuclear localization as a translational suppressor of *Fascin*. Furthermore, knockdown of *Fascin* and *Rab35* revealed gastrulation defects, consistent with the wide gut phenotype previously observed in miR-31 knockdown embryos in support of our hypothesis. Our results revealed a potential regulatory role of miR-31, Fascin, and Rab35 in early development. By identifying the targets of miR-31 and the function of its targets, our results will enable us to develop a deeper understanding of how miR-31 regulates early development and embryonic structure formation.

P782/B801

Blastocyst Injection Rescues the Postnatal Growth Retardation in Chimeric Mouse.

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While an interspecies chimeric fetus (mouse-rat) develops to a birth, its biological phenotype, such as body size, is mostly inherited from host species. Generation of donor dominant interspecies chimera would help us to understand the mechanism determining the species itself. We have recently demonstrated that high contribution of donor cells into an interspecies fetus causes an embryonic lethal, which leads to generating a low chimeric animal having the same biological features as host

species. This “xenogeneic barrier” limiting interspecies embryonic chimerism prevents a high chimeric animal from being born. Growth hormone (Gh) is one of the critical factors for postnatal body growth, which stimulates cell proliferation and metabolism. To overcome this barrier, here we aim to develop a new system which increases postnatal donor chimerism by knocking out Gh receptor (Ghr) in host embryos. **Methods**: GFP-labeled mouse embryonic stem cells (mESCs) derived from C57L/B6 strain, were prepared for blastocyst injection in basic mESCs culture condition. The Ghr gene in a mouse embryo was knocked out by electroporation with Cas9/gRNA ribonucleoprotein complexes. The mESCs were injected into wild type (WT) or Ghr knockout (Ghr KO) mouse blastocyst and then the chimeric embryos were transferred to a pseudopregnant female mouse. The body size and blood chimerism in the chimeras derived from these embryos were analyzed at several time points by flow cytometry. The chimerism in each organ was analyzed by digital PCR. **Results and Discussion:** the knockout efficiency of Ghr gene in a host embryo was over 80 % and Ghr KO mouse showed postnatal growth retardation. Compared to conventional genome editing methods, our method was rapid (over 6 months vs. 3weeks) and much efficient (25% vs. 80%). Next, we found that high contribution of WT cells into Ghr KO chimera rescued growth retardation. Besides, its blood chimerism was maintained postnatally, while WT and low chimeric Ghr KO chimeras decreased. These results indicate that the chimerism would be a critical factor determining the phenotype of chimera. The chimerism in the thymus was significantly higher in Ghr KO chimera than in WT chimeras. This result reveals that Ghr KO cells have a disadvantage in thymus niche. However, it is still unclear what factor is related to this disadvantage. **Conclusion:** We have demonstrated that Ghr KO in a host embryo provides donor cell-advantageous environment in chimera. By using this system, we would like to further generate an interspecies chimera having the same biological feature as donor species.

P783/B802

***Axin1* and *Axin2* Genes Determine Pluripotent Versus Extraembryonic Mesoderm Cell Fates in the Post-implantation Mouse Embryo.**

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The three germ layers of the mouse embryo, ectoderm, endoderm and mesoderm, are generated from the pluripotent epiblast during gastrulation and go on to form the differentiated tissues of the animal. The extraembryonic mesoderm (EEM) cell population originates in the epiblast and gives rise to tissues that facilitate maternal-embryo interaction, but the signals that regulate EEM differentiation are not well understood. In the course of studies on Axin, a negative regulator of the WNT/β-catenin pathway, we uncovered a new role for WNT signaling in the determination of extraembryonic mesodermal (EEM) cell fate. It has long been known that *Axin1* null mutant embryos exhibit partial or complete duplication of the body axis. We found that although *Axin2* single mutants are viable, *Axin1/Axin2* double mutant embryos arrest before gastrulation and the entire mutant epiblast initiates a premature epithelial-to-mesenchymal transition (EMT) and acquires an EEM identity. The expression of core pluripotency transcription factors such as Oct4, Nanog and Sox2 is downregulated in the epiblast in *Axin1/Axin2* double mutants. Embryos that lack Smad2, a mediator of signaling by Nodal recapitulate many of the defects seen in *Axin1/Axin2* double mutant embryos, including changes in pluripotency gene expression and the differentiation of the epiblast into mesoderm. This suggests that Nodal/Smad2 activation preserves the pluripotency of the mouse embryo epiblast. Epiblast-Like Cells (EpiLC) lacking *Axin1/2* genes recapitulate the defects of loss of pluripotency and premature mesoderm differentiation, facilitating the use of Nodal and Wnt pharmacological inhibitors to test the roles of these two signaling

pathways. We propose that Axin proteins regulate an instructive response of Wnt signals in the epiblast to decide between pluripotent and EEM cell fate; by contrast, Nodal signals are permissive and preserve the pluripotency and proper patterning of the epiblast. These findings define a new regulatory circuit that integrates graded WNT and Nodal/Smad2 signals to control the switch between pluripotency and EEM differentiation.

P784/B803

Distinct Prepatterns of RhoA Activity and F-actin Levels Promote Tissue Folding.

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The emergence of organism and organ shape during development requires coordinated forces that change tissue shape. One way that planar epithelia are sculpted is through bending/folding of the sheet to generate curvature, forming structures like invaginations or tubes. How epithelial sheets regulate curvature is unknown. We used the invagination of the *Drosophila* presumptive mesoderm to determine that distinct multicellular patterns of RhoA activation and F-actin levels generate proper tissue curvature. First, we showed that mechanical connections between cells are not required for graded myosin activation, suggesting that a prepattern of transcriptional activation establishes the myosin activation gradient. Second, we showed a gradient in RhoA activity that is established by proper balance between a GTPase activating protein, C-GAP and a Guanine Nucleotide Exchange factor, RhoGEF2, which establishes proper tissue curvature. Finally, we show that cells that stretch in the marginal mesoderm exhibit depleted F-actin levels compared to ventral midline cells and the ectoderm, suggesting that a zone of F-actin depletion allows cell stretching at the tissue margin to promote apical constriction and folding at the ventral midline. Thus, transcriptional patterning of contractility and F-actin levels across a tissue determines tissue curvature, which could explain the diverse shapes in different folding processes throughout development.

P785/B804

Mechanosensation of Tight Junctions by Zo-1 Phase Separation and Flow.

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Cell-cell junctions respond to mechanical forces by changing their organization and function. Tension-dependent conformational changes of junctional proteins are thought to underlie this junctional mechanosensitivity. Here we show that in the gastrulating zebrafish embryo, tight junctions (TJ) mechanosensitivity is mediated by actomyosin-driven flow of phase separated Zonula occludens-1 (ZO-1) clusters. We found that ZO-1 junctional accumulation at the contact between the Enveloping Layer (EVL) and the Yolk Syncytial Layer (YSL) closely scales with actomyosin tension. Actomyosin tension triggers ZO-1 junctional accumulation by driving retrograde actomyosin flow within the YSL that transport non-junctional ZO-1 clusters towards the TJ. Non-junctional ZO-1 clusters form by phase separation, and their effective formation is dependent on the actin binding region (ABR) within the C-terminus of ZO-1. If the non-junctional ZO-1 pool is absent, TJ lose their mechanosensitivity, and, consequently, EVL-YSL movement is impaired. Thus, phase separation and flow of non-junctional ZO-1 confer mechanosensitivity to TJ.

P786/B805

Acute Rho1 Activation Reveals That Ventral Epithelial Cells of the *Drosophila* Embryo Are Specifically Predisposed for Coordinated an isotropic Constriction during Gastrulation.

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Many morphogenetic events, including convergent extension and tube formation, require modulation of the actomyosin cytoskeleton. Ventral furrow formation in Drosophila embryos is one such morphogenetic event; it results when extracellular signals activate two transcription factors, Snail and Twist, in a subset of ventral epithelial cells. These regulators then drive the expression of multiple proteins which ultimately induce Rho1 activation, apical constriction, and invagination of cells into the embryo. These apical constrictions are anisotropic and appear coordinated, but the basis for this anisotropy and coordination is unknown. To address this and related questions, we utilized optogenetics to control Rho1 activity in the embryo. Acute Rho1 activation at the onset of gastrulation induces ectopic invaginations in both the dorsal and ventral embryonic epithelium. Rho1 activation induces apical constriction in both dorsal and ventral cells, but ventral cell constriction is stronger and more anisotropic. Strikingly, ectopic Rho1 activation induces non-cell autonomous deformations outside the activation zone only in the ventral epithelium. Thus, we demonstrate that ventral cells are specifically predisposed to respond to Rho1 activation with anisotropic and coordinated deformations. To identify the factors required for ventral cell specific behavior, we analyzed acute Rho1 activation in embryos deficient in factors required for ventral furrowing. Ventral cells depleted of RhoGEF2 exhibit anisotropic apical constriction, suggesting a molecular specialization of ventral cells beyond their ability to activate high levels of Rho1. Ventral cells lacking Twist exhibit weaker and less anisotropic apical constrictions, indicating a previously unknown role of Twist. Unlike wildtype or twist embryos, Rho1-induced deformations persist in RhoGEF2-depleted embryos. Collectively, our results demonstrate that while Rho1 is sufficient to initiate invagination throughout the embryonic epidermis, the individual cell shape changes accompanying these invaginations differ between dorsal and ventral cells. Additionally, ventral cells specifically can propagate the response to Rho1 activation outside of the zone of optogenetic activation. Experiments are in progress to identify the factors required for these ventral specific behaviors.

P788/B807

A *Drosophila* Deficiency Screen for Genes on Chromosome 2L Involved in Dorsal Closure Using a Live Imaging Approach.

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Cell sheet morphogenesis is essential for metazoan development and homeostasis of animal form - it contributes to developmental milestones including gastrulation, neural tube closure, heart and palate formation as well as tissue maintenance in wound healing. Dorsal closure occurs during *Drosophila* embryogenesis and has emerged as a model for cell sheet morphogenesis throughout phylogeny. Closure is a remarkably robust process where conserved gene expression and signaling cascades are coordinated to regulate the cellular machines that drive closure. Many of the ~140 known 'dorsal closure genes' were identified in screens by scoring for a terminal, dorsal open cuticle phenotype. Thus, key genes that contribute to the kinematics and dynamics of closure may not have been identified.

Here, we extend our previous study of the right arm of the 2nd chromosome (2R, Mortensen *et al*. 2018 G3: Genes|Genomes|Genetics 8:2361) to the left arm of the 2nd chromosome (2L). We used the Bloomington 2L deficiency kit, a set of large deletions, which collectively remove 98.9% of the genes on 2L to identify 'dorsal closure deficiencies'. Through two crosses, we unambiguously identify embryos homozygous for each deficiency and time-lapse image cell shapes for the duration of closure. Confocal images of GFP-cadherin are analyzed for defects in cell shapes and tissue movements. We have successfully screened 96.1% of the genes on 2L and identified embryos homozygous for 46 deficiencies with notable, diverse defects in closure. Of these, 26 have no known dorsal closure gene removed by the deficiency region. These include defects in cell shape, canthus formation and tissue dynamics. As with our previous analysis of the 2R, we anticipate further analysis of these and other 2L deficiencies will lead to the identification of novel 'dorsal closure genes' (for example *pimples* accounts for dorsal closure defects seen in embryos homozygous for Df(2L)63). We expect to identify links between pathways and structures already known to coordinate various aspects of closure as well as new processes and pathways that contribute to closure. Grant Support: R35GM127059 to DPK; NSF-DGE 1644868 to SMF; T32 GM007184 to SMF, RPM and RDM.

P789/B808

TAF13 Is Essential for Early Mammalian Development.

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TATA-Binding Protein Associated Factor 13 (TAF13) is one component of the transcription pre-initiation complex (PIC). The transcription PIC is essential for initiation of the transcription of protein-coding genes. The transcription PIC is made up of the RNA polymerase II (RNA pol II) and six general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH. The general transcription factor TFIID is made up of TATA-Binding Protein (TBP) and 13 highly conserved TBP-Associated Factors (TAFs). We have undertaken a systemic characterization of TAF13 homozygous mutants in order to determine the developmental and molecular phenotype in embryos lacking functional TAF13. Breeding TAF13 +/- mice does not yield TAF13 -/- pups, indicating that the TAF13 -/- embryos are dying in utero. Our studies reveal that TAF13 -/- embryos are developmentally delayed compared to their littermates by day 7.5 of embryonic development (E7.5) and fail to initiate gastrulation. We also show that key developmental transcription factor expression is absent in these mutant embryos, showing for the first time that TAF13 is required for TFIID/PIC function in vivo.

P790/B809

Stretch Forces Activate Mechanically-gated Calcium Channels Elevating Calcium and Nitric Oxide in the Ectoderm Germ Layer of the Chicken Embryo.

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Early embryo development for myogenesis requires morphogen inductive signaling over the paraxial mesoderm that results in somite formation, its compartmentalization, and myotome and sclerotome tissue formation. While morphogens have been well studied in embryonic development, other molecular regulators have not, including the pleiotropic signaling molecule, nitric oxide (NO). We previously reported that calcium (Ca2+) and NO signals are co-localized in the ectoderm layer over the embryo midline and somites creating a pattern to correlate with nascent myotome formation. However, how these ectoderm signals are initiated is unknown. We propose that rapid embryonic growth and

development are possible physical activators of Ca2+ and NO signals through a strengthening mechanical stretch force on the ectoderm layer due to a rapidly growing neural tube at the cranial embryo location. To investigate mechanical force activation of ectoderm Ca2+ and NO, we fabricated a custom made “rake” device to control tension by medial-lateral stretching of the area opaca in HH10 staged embryos. Live-imaging through spinning disc confocal microscopy was used on embryos labeled for Ca2+ using calbryte™520 and NO using DAF-2. Our results show pulls of the area opaca of 0.5 mm resulted in 2-fold elevations of ectoderm Ca2+ within 3-seconds and a return to Ca2+ baseline levels at the embryo midline and over cranial located somites. To determine if mechanically-gated Ca2+ channels produce the Ca2+ rise on applied tension, whole embryos were treated with 250 µM of gadolinium chloride (GdCl3) that inhibited Ca2+ elevation to an only slight increase. Furthermore, embryos in 1mM EGTA to deplete medium Ca2+ levels were stretched and produced no Ca2+ rise in the ectoderm. However, a subsequent rise in NO could not be produced with a momentary rise of ectoderm Ca2+ under our stretch conditions. In contrast, ectoderm isolated as sheets of cells, cultured for 1 day on stretchable PDMS membrane, could produce a rise in Ca2+ and a later increase in NO over 30 minutes of stretch. In conclusion, the ectoderm under stretch activates opening of a mechanical-gated Ca2+ channel that can produce a NO elevation in culture conditions. We anticipate NO to elevate as well in whole embryos with longer times of Ca2+ elevation involving activation of other Ca2+ channels, like the L-type channels. **NSF STC CCC: 1548297**

Stem Cells

P791/B810

Tristetraprolin (ttp) Expression Is Required for Maintenance of the Mammary Progenitor Cell Population.

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Messenger RNA (mRNA) stability is regulated mainly by proteins that bind sequences enriched in adenine and uracil in their 3´untranslated regions, called collectively AU-binding proteins (AUBPs). Tristetraprolin (TTP) is an AUBP that promotes mRNA degradation of proteins involved in inflammation, proliferation and tumor invasiveness. We have previously reported that TTP expression is down-regulated in breast cancer compared to normal mammary gland, where TTP reaches its highest levels during lactation. In addition, bitransgenic females WAP-Cre x $TTP^{fl/fl}$ mice (named MG TTP-KO), showed that reduction of TTP levels in the lactating mammary gland resulted in premature involution associated to the increase of TNFα, IL-6 and LIF expression, STAT3 activation and massive cell death. Therefore, we concluded that TTP expression in the mammary epithelium is required for lactation maintenance. Then, as it has been reported that WAP-expressing cells behave as a pregnancy-induced mammary progenitor subpopulation and analysis of RNA-seq data indicates that TTP is up-regulated in mammary progenitor cells, our next goal was to determine whether expression of this AUBP is relevant for the maintenance of the mammary stem cell compartment. Our results showed that upon successive pregnancies MG TTP-KO mice exhibited underdeveloped lactating glands that also presented decreased pre-neoplastic lesions

when crossbred with WAP-Cre/RasG12D mice. Moreover, mammary glands from total TTP-KO mice displayed underdeveloped ductal network upon transplantation into syngeneic mammary cleared fat pads. This suggested that diminished expression of TTP in the mammary progenitor compartment caused impairment of mammary gland development and differentiation. To verify this hypothesis, we generated TTP knockdown cells (TTP-KD) by stable transfection of stem-like HC11 mammary epithelial cells with specific TTP-shRNA constructs. Interestingly, these cells exhibited impaired survival rates (by MTS analysis), increased apoptosis (by TUNEL), and induction of pro-apoptotic proteins (by Western blot, WB). Besides, TTP-KD cells also displayed high expression of inflammatory cytokines (analyzed by RT-qPCR), increased levels of STAT3 and P-STAT3 as well as p65/RelA and p38 phosphorylation, but inhibition of ERK 1/2 activation (studied by WB and treatment with specific inhibitors). Importantly, the TTP-KD cells revealed a substantial decreased capacity to form mammospheres in 3D culture and to repopulate cleared fat pads of virgin BALB/c female mice. Taking together, our results indicate that TTP expression is required for mammary progenitor cell survival by preventing spontaneous pro-inflammatory and stress-associated events, which are able to induce mammary stem cell death.

P792/B811

Comparative Single Cell Transcriptomic an alyses Reveal Molecular Similarities and Differences of Stem Cell Biology during Mammary Gland Development and Regeneration.

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A central goal in vertebrate stem cell biology is to understand the molecular basis of crosstalk between stem cells and their niche microenvironment during development, regeneration, and pathology. Here, we have used single cell transcriptomics and genetic manipulations to interrogate stem cell - niche interactions during differentiation and morphogenesis at various phases of mammary gland development and regeneration. We found that, during mammary gland regeneration, adult stem cells are “reprogrammed” to an embryonic progenitor state, followed by subsequent differentiation stages similar to those during development. This has allowed by to identify candidate genes and signaling pathways that regulate critical stages of mammary stem cell differentiation. Furthermore, we found that FGF signaling not only plays an essential role between stem cell and stromal microenvironment, it is also essential for luminal and basal interactions during differentiation and epithelial branching. These data thus highlight the complexity of the cellular components of stem and niche cells and underscore the pluripotent roles of FGF signaling in regulating their interactions.

P793/B812

Defects in Neural Precursor Proliferation and Abnormal *Asxl1* Expression as a Result of Mutations in *Hcfc1* Ortholog Induce Hypoactive Behavior in Zebrafish Model of *Cblx* Syndrome.

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Methylmalonic acidemia and homocysteinemia, clbX type (*cblX*) (309541) is rare multiple congenital anomaly syndrome caused by mutations in the *HCFC1* gene which encodes for a transcriptional cofactor. The neurological phenotypes associated with *cblX* are profound, yet the mechanisms by which mutations in *HCFC1* cause such deficits remain unknown. This developmental disease is characterized by severe neurological impairment, indicating a function for HCFC1 during normal brain development. Previous studies suggest that HCFC1 regulates neural precursor cell (NPC) proliferation and differentiation. However, HCFC1 regulates a diverse array of target genes and has been shown to bind to

the promoters of more than 5000 unique downstream target genes. Consequently, the molecular mechanisms by which HCFC1 regulates NPC proliferation and differentiation are complex. Therefore, we hypothesize that HCFC1 is a master transcriptional regulator of NPC function. To test this, we have developed a zebrafish harboring a germline mutation in *hcfc1a*, a zebrafish ortholog of *HCFC1*. Using this platform, we discovered that *hcfc1a* regulates the proliferative state of NPCs in *vivo* by mediating the developmental expression of *asxl1*, a polycomb transcription factor. Furthermore, we found that the additional NPCs that are generated continue through the differentiation pathway and produce excess neurons and glia. Importantly, these excess differentiated cells were associated with hypo-locomotive behavioral deficits. Collectively, our data provides a novel mechanistic insight into the transcriptional program modulating NPC proliferation, brain development and motor function.

P794/B813

Notch Signaling Regulates Neural Stem Cell Entry into Quiescence in *Drosophila*.

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During development, neural stem cells divide in a symmetric manner to expand the stem cell pool and in an asymmetric manner to generate different types of progeny. Neural stem cells also switch between periods of quiescence versus proliferation in response to both intrinsic and extrinsic factors. One important extrinsic factor regulating proliferation decisions is the availability of nutrients because dietary nutrients provide the building blocks necessary to fuel the cell divisions. We are interested in understanding how quiescence versus proliferation decisions are controlled during development. We use *Drosophila* as a model system because of the availability of genetic tools and because the population of neural stem cells, known as neuroblasts in *Drosophila*, is relatively simple and defined. Most neuroblasts in the central brain enter and exit quiescence in a nutrient-dependent and PI3-kinase regulated manner. To better understand how neural stem cell proliferation decisions are regulated we carried out a large-scale RNAi screen. From this screen, we identified components of the Notch signaling pathway. Notch signaling is an evolutionarily conserved juxtacrine cell signaling pathway that in the *Drosophila* central brain allows for cross-talk between both neuroblasts and their daughters and between neuroblasts and their glial niche. To activate Notch signaling, Notch receptor binds to its ligand, Delta. Here we investigate how Notch/Delta signaling regulates neuroblast entry and exit from quiescence during the embryonic to larval transition. We found that Notch signaling in neuroblasts is activated in a nutrient-dependent manner and once on, it remains on in a nutrient-independent manner. Next, we used RNAi to knockdown Notch signaling in neuroblasts. After 24hrs of feeding, we found no difference in neuroblast proliferation compared to control. However, in freshly hatched larvae before animal feeding, we found ectopic neuroblast proliferation when Notch or Delta was knocked down. This suggests that Notch/Delta could be required for neuroblast entry into quiescence. To investigate this possibility, we assayed neuroblast proliferation prior to neuroblast entry into quiescence. We found that neuroblast proliferation continued longer when Notch or Delta was knocked down in neuroblasts compared to control. Next, we assayed the expression of Notch signaling components as neuroblasts entered into quiescence. We found that Notch and Delta expression was downregulated as neuroblasts enter quiescence. We conclude that Notch signaling is required for neuroblast entry into quiescence and that active Notch signaling correlates with neuroblast proliferation.

P795/B814

Understanding the Role of *LAMB4* in the Neurodegenerative Disease Familial Dysautonomia.

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The genetic childhood disorder Familial Dysautonomia (FD) is a particularly devastating disease of the peripheral nervous system (PNS). Patients present with a range of symptom severity; however, the mechanism behind this phenomenon is unknown. Patients with FD harbor a missense mutation in exon 19 of the gene *IKBKAP*, resulting in low IKAP protein levels in PNS neurons, which causes impaired development and survival of sensory and autonomic neurons. Although FD is a monogenetic disease, patients exhibit a wide range of disease severity, such as mildly or severely decreased ability to sense pain and different frequency of dysautonomic crisis. The recently established mouse model for FD does not completely recapitulate the human disorder, particularly with respect to severity differences. Thus, its predictive value is limited. Similarly, due to lack of access to cells targeted by the disease (i.e., sensory neurons), the molecular knowledge we have of FD pathophysiology is limited and may not translate to what occurs in physiological conditions. Therefore, the mechanism underlying the causes of FD severity is not understood. Human pluripotent stem cells represent a powerful platform to study FD in a disease-relevant and tissue-specific context. We previously used induced pluripotent stem cells (iPSCs) derived from FD patients to model disease severity. We found that restoring the mutation in *IKBKAP* did not rescue all severe FD phenotypes, suggesting that other factors might be involved in FD severity. Agreeing with this, we found that FD patients with severe symptoms harbor a mutation in *LAMB4* in addition to *IKBKAP*, that is absent in mild FD patients. Thus, I hypothesize that *LAMB4* is necessary for normal PNS development and loss of wild type *LAMB4* exacerbates the symptoms to severe FD in patients. Agreeing with this, I found that SNs from patients with severe FD exhibit low levels of lamninin-β4 (the product of *LAMB4*) compared to healthy controls. The exact function of *LAMB4* is unknown. However, *LAMB4* mutations have been associated with the enteric neuropathy Diverticulitis, supporting my hypothesis that *LAMB4* plays a role in neurodevelopment and in diseases of the PNS. My studies will provide insight on the role of *LAMB4* during development of the PNS. Moreover, it will uncover the mechanism behind the symptom severity observed in FD, providing potential new approaches for the development of novel therapeutics.

P796/B815

Netrin-1 Synthesized in Satellite Cells Isolated from Fast-twitch Muscles May Promote Fast Myofiber-type Commitment.

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Resident myogenic stem cells, satellite cells, are indispensable for successful regeneration and hypertrophy on skeletal muscle fibers (myofibers). We currently focus on a novel role of satellite cells in the myofiber-type regulation. Myofibers can be classified into slow- or fast-twitch muscle based on colors, contractile properties and metabolisms. Our previous studies showed that satellite cells prepared from soleus muscle (slow-myofiber abundant) synthesize and secrete larger amount of semaphorin 3A (Sema3A, a multi-functional protein originally found as a neural chemorepellent) than extensor

digitorum longus (EDL; fast-fiber abundant) at myogenic differentiation phase (Suzuki et al. 2013). The subsequent study demonstrated that Sema3A impacts the formation of slow-twitch myotube through the cell-membrane receptor complex neuropilin2/plexinA3 signaling pathway at the early-differentiation phase after muscle injury(Tatsumi et al. 2017). In contrast to this Sema3A-driven mechanism, there are few knowledges about regulatory mechanisms for “fast-twitchmyotube commitment” by a secretory protein(s) from satellite cells. In this study, we hypothesized the function of Netrin families (Netrin-1, 3 and 4) that may contribute to fast-twitch myotube formation since their physiological significances are known to compete with Sema3A in neurogenesis and osteogenesis. To evaluate this hypothesis, we examined whether Netrins impact fast-twitch myotube generation through the expression profiles of Netrin families in primary cultures of satellite cells and knock-down by specific siRNAs transfection with RNA interference technique. We revealed that Netrin-1 and 4 were significantly up-regulated like a Sema3A expression pattern during myogenic differentiation phase. Subsequently, we compared the expression levels of Netrin subtypes and receptors between soleus- and EDL-derived satellite cells; there were no significant differences in expression levels of Netrin-3, 4 and Netrin’s cell-membrane receptors (neogenin, BOC, CDO, Unc5B and 5C), while satellite cells from EDL expressed higher levels of Netrin-1 than those from soleus. Moreover, Netrin-1 knock-down inhibit myotubes formation by significantly diminishing the expression level of fast-type myosin heavy chain (especially in type IIb). These findings suggest that Netrin-1 synthesized in EDL-derived satellite cells would act as a key modulator to promote myofiber-type commitment of fast muscles. This work was supported by JSPS KAKENHI and the grant fund from the Ito Foundation.

P797/B816

Isolation and Characterization of Putative Skeletal Stem Cells from the Effluent of Autologous Bone Graft Preparations.

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The recently identified human skeletal stem cells (hSSCs) are essential for maintaining bone homeostasis and for promoting bone repair. Therefore, they present a tremendous potential for the treatment of many orthopaedic applications. However, a major bottleneck is the limited sources for these rare cells. A common method for the harvest of autologous bone graft is the Reamer Irrigator Aspirator (RIA) system, where the intramedullary canal of a long bone (often femur) is reamed, irrigated and aspirated with a filter trap to collect semi-solid stroma/bone material used for grafting procedures. During this procedure, a large volume of liquid and small particles is discarded as waste. Here we show that this waste fluid (RIA effluent), is rich in CD45-CD51+CD200+ cells, which are putative hSSCs. Using single cell RNA sequencing, we demonstrate that this cell population is quite homogeneous, with differentially expressed genes associating with mitochondrial genes and cell signaling. In immune deficient mice undergoing bone fracture, intramuscular injection of hSSCs leads to a significant improvement in bone repair as shown by microCT and calcein staining. Of note, we show that these cells home preferentially to bone and bone marrow after intravenous injection, suggesting that the cells may be applicable to treat systemic skeletal defects, such as osteoporosis. Altogether, our data shows that RIA effluents are a rich source of potential hSSC, which could lead to the development of new regenerative medicines for orthopedic applications.

P798/B817

Gastric Cell Proliferation and Stem Cells Niche: Effects of Early Weaning.

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Gastric epithelial cells proliferate at the isthmus-neck region of the gland, and this interface is considered the main stem cell niche. In adult animals, an additional inactive- reserve niche is also found at gland base. Such arrangement of proliferative compartment is achieved at weaning time in rats, but the characterization of markers for stem/progenitor cells is still under discussion and little is known about postnatal development. Considering that breastfeeding is important for development program, our hypothesis was that early weaning (EW) might modify the expression of genes that control stem cell activity, and consequently would alter the growth and maturation of stomach. Our aims were to evaluate the immediate and late effects of EW in cell proliferation, at expression genes related to stem cell niche and identify Troy-cell (quiescent stem cell marker) population and distribution in the gastric gland in rats. Wistar rats were submitted to EW (15d) and gastric samples were collected at 15d, 18d and 60d for RT-qPCR, tissue and cell morphology (IHC-P), and immunofluorescence (Ethics Committee CEUA ICB USP 18/2015; 115/2017). We observed that EW augmented proliferation index and thickness of mucosa at 18d, whereas at 60d, Ki-67 immunolabeling variations were not detected. As for genes involved in stem cell niche, EW increased *Tnfrsf19* (Troy) at 18d, but differences were not found at 60d when compared to control (suckling - S). *Notch1* and *Notch2* expression decreased after EW at 18d whereas, both of them increased at 60d. Additionally, *Bmp2* expression was higher at 18d and *Axin2* at 60d. Our results also indicated that in S, at 18d, Troy+cells were found along gland extension, and in the EW group, they were restricted to the isthmus-neck region. At 60d, Troy+-cells were found at the base in S group, whereas it was more spread after EW. We used multiple labeling to study the co-localization of Troy with other epithelial markers and observed that Troy+-cells did not correlate with Ki-67-proliferative cells, but they double-stained with H^+K^+-ATPase, indicating that parietal cell expressed Troy at both S and EW groups at all ages evaluated. Our results suggested that although Troy+ cells do not co-localize with Ki-67-proliferative cells during development, they are sharing the same area, indicating that Troy+ cells are part of the proliferative niche. Additionally, in S adults, we observed Troy+ cell in the quiescent niche, whereas in EW group, its distribution was not restricted to one site. Furthermore, we observed that after EW the expression of genes involved in signaling and stem cell niche behaved differently over aging, and so, the premature dietary change influenced the proliferative compartment.
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P799/B818

Estrogen Supplement Enhances the Endothelial Differentiation in Stromal Cells Derived from Adipose Tissue of Rats with Ovariectomy.

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The decrease inquantity and quality of stem cells is found to be associated with age, particularly after menopause. The estrogen level is supposed to have an influence on the performance of stem cell therapy in regenerative medicine. The aim of present study is to determine whether estrogen supplement has benefit in promoting endothelial differentiation of adipose-derived stromal cells (ADSC) and enhancing its angiogenic activity. To examine the effect of estrogen treatment on activating estrogen signaling and promoting endothelial differentiation in ADSC, adipose stromal cells were

isolated form adult male rats and cultured in EGM2 medium to induce endothelial differentiation, followed by Western blotting and immunofluorescent staining. Results showed that both expression levels of estrogen receptor α (ERα) and β (ERβ) were increased after estradiol (E2) treatment. Of interest, ERα was localized in the cytoplasm, while ERβ was observed in both nucleus and cytoplasm. In addition, the activation of membrane-initiated estrogen signaling downstream proteins, PI3K signaling, was increased in ADSC cultured with E2 supplement. Furthermore, the phosphorylation level of MAPK signaling proteins, including p38 and JNK, were also increased with E2 treatment. In vitro matri-gel assay showed that the tube and network formation activity of ADSC was up-regulated by E2 treatment. RT-PCR results demonstrated the mRNA expression level of eNOS and VE-cadherin, two indices of functional endothelial cell, andHGF, a pro-angiogenic growth factor, were increased in ADSC with E2 treatment. Results from Western blottings also indicated that protein expression levels of eNOS and VEGF in ADSC were up-regulated by E2 treatment. Flow cytometric analysis further demonstrated that the level of cells positively stained with endothelial cell markers, including CD34, KDR, and CD31 were increased in ADSC cultured with E2. An imal studies also indicated that the number and angiogenic activity of ADSC with endothelial differentiation were reduced by ovariectomy in adult rats, while the impairment of ADSC was rescued by estrogen supplement. In conclusion, the endothelial differentiation of ADSC was enhanced by E2 treatment in vitro and in vivo. while of activation of PI3K and MAPK-mediated estrogen signaling was observed in E2-treated ADSC.

P800/B819

High Mannose N-glycans Promote Migration of Bone Marrow-derived Multipotent Stromal Cells.

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Human bone marrow-derived multipotent stromal cells (MSCs) are currently being tested for hundreds of indications, but often therapeutic efficacy has not been achieved due to an inability of the cells to reach target tissues. Here we show that inducing high mannose N-glycans either chemically using the type I mannosidase inhibitor Kifunensine, or genetically using an shRNA to silence expression of mannosidase I A1 (MAN1A1), strongly improve the migration potential of the cells, as determined using wound/scratch assays and videomicroscopy. This increase in high mannose N-glycans also improves active cell migration toward bone fracture after percutaneous injection, and passive cell migration toward lungs after intravenous injection, in immune deficient mice. Mechanistically, high mannose N-glycans induce a change in cell shape, reducing the area in contact to substrate and increasing cell height, as measured using atomic force microscopy. We also show that a reduced contact area strongly correlates with increased cell motility. To determine if pre-treatment with Kifunensine is feasible for future clinical studies, we used NanoLC/ESIQTOF-mass spectrometry to study the N-glycan profile of MSCs over time and demonstrate that the effect of Kifunensine is transitory. We also describe the effect of this compound on cell proliferation, differentiation, and on the secretion profile of MSCs. Altogether, our results support the notion of pre-conditioning MSCs to induce high mannose N-glycans, which leads to increased motility of the cells.

P801/B820

Elucidating the Cell Mechanics That Underlie Stem Cell Niche Morphogenesis.

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Many of our tissues are maintained and renewed by stem cells. These cells rely on signaling cues from the niche in which they reside. Growing evidence in several systems suggest that the precise organization of niche cells influences how efficiently they control stem cell behavior. Thus, understanding niche morphology will provide insight into how stem cells are maintained. We use the gonad stem cell niche in *Drosophila* to study the mechanics of niche formation. This model combines genetic tractability with powerful live-imaging techniques pioneered in our lab. Our live-imaging has shown dynamics of niche formation during two phases: 1) **assembly** and 2) **compaction**. During **assembly**, pro-niche cells first migrate towards the gonad anterior, gathering in an elongate mass. During **compaction**, the pro-niche cells reorganize forming a smooth, circular contour linked by adherens junctions to its stem cells. These stem cells divide perpendicular to the niche, in order to place daughter cells outside the influence of self-renewing signals. We hypothesize that a smooth contour is crucial to induce properly oriented divisions. I am interested in unraveling cytoskeletal mechanisms that affect compaction and thus affect stem cell function. We show that F-actin and Myosin II (Myo-II) are enriched at stem cell-niche interfaces during compaction. In addition to the enrichment of F-actin and Myo-II, we have shown that there are tensile forces along stem cell-niche interfaces. I hypothesize that these forces are due to actomyosin contractility along niche-stem cell adherens junctions, which function in compacting the niche. I am currently testing this hypothesis by genetically disrupting cytoskeletal modulators and assaying compaction through live imaging. This work will be among the first to describe the mechanisms of shaping a functional niche, and will bridge an important gap in stem cell biology.

Protists and Parasites

P802/B822

Ploidy Cycles as a Strategy of the Advantage from Unicellular Eukaryotes to Mammalian Cancer Cells.

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Normal 0 false false false RU X-NONE X-NONE /* Style Definitions */ table.MsoNormalTable {mso-style-name:"Обычная таблица"; mso-tstyle-rowband-size:0; mso-tstyle-colband-size:0; mso-style-noshow:yes; mso-style-priority:99; mso-style-qformat:yes; mso-style-parent:""; mso-padding-alt:0cm 5.4pt 0cm 5.4pt; mso-para-margin:0cm; mso-para-margin-bottom:.0001pt; mso-pagination:widow-orphan; font-size:11.0pt; font-family:"Calibri","sans-serif"; mso-ascii-font-family:Calibri; mso-ascii-theme-font:minor-latin; mso-fareast-font-family:"Times New Roman"; mso-fareast-theme-font:minor-fareast; mso-hansi-font-family:Calibri; mso-hansi-theme-font:minor-latin; mso-bidi-font-family:"Times New Roman"; mso-bidi-theme-font:minor-bidi;} It is considered that the absence of sexual process results in accumulation of harmful mutations in the sequence of generations of obligate agamic organisms - the so-called “Muller’s ratchet”. Therefore, there must be the mechanisms of “zeroing” this negative effect in the life cycle of lower agamic eukaryotes. According to one of hypotheses the cyclic polyploidy (alternation of de- and polyploidization in the course of life cycle) may play the role of such a mechanism. We have shown that *Amoeba proteus*, a textbook agamic unicellular eukaryote, and related

amoebae species, employ a special kind of cyclic polyploidy, achieving depolyploidization by chromatin extrusion from the nucleus into the cytoplasm. The phenomenon of “extra-DNA” extrusion from the nucleus during the cell cycle was found in some representatives of multicellular organisms from the macrotaxae which were far from each other, and had no phylogenetic connections; in unicellular eukaryotes it was found in macronuclei of some ciliates. It means that the cell mechanisms of nuclear extrusion are rather ancient and universal. Surprisingly, the similar strategy of cell cycle was shown for cancer mammalian cells after genotoxic treatment, in this case it may result in their survival, and, even in increasing of tumor aggressiveness. Thus, our data on amoebae sustain the so-called “atavistic theory of carcinogenesis”. Moreover, the detailed study of those lower eukaryotes whose cell cycles include the strategy of cyclic polyploidization (*Amoeba proteus* among them) may serve the new tool for understanding the roots and mechanisms of high cancer cells stability to genotoxic treatment. This work was partly financed by the Budgetary Program # 0124-2019-0005 at the Institute of Cytology RAS.

P803/B823

The Dark LECA Rises: an cient Proteins with Unknown Functions.

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Reconstructing the cellular systems of the Last Eukaryote Common an cestor (LECA) is an ongoing challenge. It is commonly accepted that LECA had all of the general features that extant eukaryotes share including a nucleus, mitochondria, flagella, a complex cytoskeletal and membrane trafficking systems, the capacity to engulf prey, and facultative sex. Although these general features of LECA are known, the degree to which LECA is a mystery remains mysterious. An cestral reconstructions are limited by our cell biological knowledge of model organisms and the degree to which functional investigation in models might contribute to human health. This leaves a vast diversity of ancient genes neglected for which no functional information is available. In order to identify ancestral orthologue groups with little or no structural or functional information, we used sequence clustering approaches, tree building, and manual curation to identify two types of ‘dark’ genes in LECA. First, the dark proteome, which comprises genes encoding proteins for which no domain information is available. Second, the dark paralogues, which comprise genes encoding proteins for which domain information is available their precise functions remain unknown. We suggest that between ~10-30% of genes in LECA lack functional or structural annotations leading to the conclusion that our knowledge of the cell biology of the eukaryote ancestor is significantly lacking.

P804/B824

Structural Studies of the Microsporidian Polar Tube.

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Microsporidia are obligate intracellular, eukaryotic parasites capable of infecting a wide range of hosts from silkworms to immunocompromised humans. They do so through a magnificent organelle known as the polar tube which undergoes tremendous conformational change from being tightly coiled inside the dormant spore to a long, linear tube out of the spore during infection. The filamentous polar tube is

likely composed of repeating units of polar tube proteins (PTPs) whose structures remain a mystery, and which also don't share sequence homology to any known protein. Furthermore, it is not well understood how these proteins are arranged to form the tube. An understanding of the protein structures, as well as how they are arranged to potentially form repeating units and information on the ultrastructure of the tube will be key to understanding the invasion mechanism. We have optimized sample preparation for cryo-tomography and observed two different populations of the polar tube of microsporidia species *Encephalitozoon hellem*. One population contains stripes diagonal to the length of the tube while the other population contains layers which give it a leek like appearance, and these may represent two stages of the polar tube firing process. We are currently trying to perform subtomogram averaging to improve the resolution of our model and optimizing recombinant polar tube proteins for x-ray crystallography. To understand the composition of the tube through proteomics, we have also optimized a method for shearing the polar tube off the germinated spore using sonication which omits the need to use harsh chemicals traditionally used in polar tube extraction. By taking advantage of the advances made in cryo-electron tomography and combining it with crystallography and proteomics we hope to understand how the architecture of the polar tube gives it robust material properties and facilitates infection.

P805/B825

Ensnared Dynamics of Miniature Mitochondrial Organelles.

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Division of the eukaryotic cell is a coordinated process, during which the daughter cells receive, in addition to the nucleus, a set of organelles to maintain all cellular functions. While some organelles like peroxisomes or even Golgi complex can be generated de novo, mitochondria can only bepropagated from the pre-existing organelles. Here, we show that a unicellular eukaryote (protist) *Giardia intestinalis*, earmarked a "germline" set of its mitochondrial organelles known as mitosomes, to ensure their faithful propagation into the daughter cells. These mitosomes divide within secondsonly during the early prophase of mitosis and they are distributed equally with the half set of the cell flagella to daughter cells. Surprisingly, this mitochondrial division occurs even when mitosis is blocked, which results into formation of anucleate cells but with a proper set of mitosomes andflagella. This is achieved by physically connecting "germline" mitosomes to the axonemes of the cell flagella via a specialized lamella made of a sheet of microtubules. The 3D models of the whole mitotic cells generated by FIB/SEM microscopy demonstrate that it is always only the oldest pair of eightflagella to which the mitosomes are ensnared. We propose that the flagellar system controls the segregation of mitochondrial organelles also in other members of this supergroup of eukaryotes. Moreover, the integration of mitochondrion into the flagellar system could have been the originalstrategy of the early eukaryotic cell to maintain its key organelle.

P806/B826

Characterization and Evolution of the Polar Tube of Microsporidia - the Invasion Apparatus of the Intracellular Parasites.

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Microsporidia are obligate intracellular pathogens, which infect wide spectrum of hosts ranging from protists to vertebrates including humans. Key to the infection is the penetration of the parasite into the host cell [1]. Microsporidia carry unique invasion apparatus built around a hollow proteinaceus tube (polar tube), which is ejected from the infectious spore and allows the parasite to be injected into the host [2]. Polar tube can be as long as 500 micrometers and is built of at least three different proteins (PTP1-3). We have been studying the localization and the interaction of PTPs within the tube in order to understand its biogenesis and structure on a model of silkworm pathogen *Nosema bombycis*. Using super resolution and cryo electron microscopy we have showed that the polar tube is a protein polymer of 100nm in diameter with a periodic pattern of 63 angstroms. Interestingly, this pattern seems to be conserved across different species of Microsporidia despite the great difference in the primary structure of all PTPs [3]. Using the bioinformatic analyses we have mapped the evolution of the polar tube and PTPs across Microsporidia and defined a core set of PTPs, which is common to all species. 1. Weiss, L. M. & Becnel, J. J. *Microsporidia: Pathogens of Opportunity: First Edition*. (Wiley Blackwell, 2014). doi:10.1002/9781118395264 2. Lom, J. & Vávra, J. The mode of protoplasm extrusion in microsporidia spores. *Acta Protozool* 81-92 (1963). 3. Takvorian, P. M. *et al. An* Ultrastructural Study of the Extruded Polar Tube of an *ncaliia algerae* (Microsporidia). *J. Eukaryot. Microbiol.* jeu.12751 (2019). doi:10.1111/jeu.12751

P807/B827

Microsporidia Harpoon-like Polar Tube, from Firing Dynamics to Host Cell Entry.

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Microsporidia are obligate intracellular parasites that infect a variety of invertebrate and vertebrate hosts including humans. Microsporidia employ a unique invasion organelle, the polar tube, that forms a coil resembling a spring inside dormant spores. Under suitable environments, microsporidia fire the polar tube, which extends from its coiled structure to a linear tube, to puncture the host cell membrane and serves as a tunnel for the transferring of the parasite's infectious material. This firing process occurs extremely fast, on the millisecond timescale. Invasion of host cells using the polar tube is thought to be the dominant mechanism of host cell entry. In addition, phagocytosis of whole spores has also been reported to be an alternative route of entry in some microsporidian species. However, little is known about the mechanism of entry, either via the polar tube or via phagocytosis. Here, we optimized high-speed, live-cell optical imaging to capture the firing events from two microsporidian species, an *ncaliia algerae*, which can infect mosquito's gut and human muscles, and *Encephalitozoon hellem*, which mainly infects eye tissue. We find differences in the firing kinetics between the two species, which may be linked to their tissue specificity. To investigate how our in *vitro* studies may be applicable in the

presence of host cells, we developed a fluorescence microscopy-based assay that allowed us to investigate PT-mediated and phagocytosis-mediated entry in host cells. While PT-mediated entry is dominant in *A. Algerae*, phagocytosis-mediated entry is dominant in *E. hellem*. Host cell actin cups form around intact spores during phagocytosis, and also around the naked sporoplasm that enters the host cell via PT-mediated infection. We will discuss the dynamics of the large PT organelle in *vitro*, as well as the microsporidia invasion and entry process in the presence of host cells.

P808/B828

***Invitro* Activity and Mode of Action of Phenolic Compounds on *Leishmania Donovani*.**

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Background: Leishmaniasis is a disease caused by the protozoan parasite, *Leishmania. The* disease remains a global threat to public health requiring effective chemotherapy for control and treatment. In this study the effect of some selected phenolic compounds on *Leishmania donovani* was investigated. The compounds were screened for their anti-leishmanial activities against promastigote and intracellular amastigote forms of *Leishmania donovani.* **Methodology/Principal findings**: the dose dependent effect and cytotoxicity of the compounds were determined using a tetrazolium-based assay. Flow cytometry was used to determine the effect of the compounds on the cell cycle. Parasite morphological analysis was done by microscopy and growth kinetic studies were conducted by culturing cells and counting at 24 hours intervals over 120 hours. The iron chelating property of the selected compounds was determined by atomic absorption spectroscopy and the effect of compounds on the expression of iron dependent enzymes was investigated using RT-qPCR. The IC_{50} of the compounds ranged from 16.34 µM to 124 µM compared to amphotericin B and deferoxamine controls. Rosmarinic acid and apigenin were the most effective against the promastigote and the intracellular amastigote forms. Selectivity indexes (SI) of rosmarinic acid and apigenin were 15.03 and 10.45 respectively for promastigotes while SI of 12.78 and 5.20 respectively were obtained for intracellular amastigotes. Morphologically, 70% of rosmarinic acid treated promastigotes showed rounded morphology similar to the deferoxamine control. About 30% of cells treated with apigenin showed distorted cell membrane. Rosmarinic acid and apigenin induced cell arrest in the G_0/G_1 phase in promastigotes. Elevated intracellular iron levels were observed in promastigotes when cells were treated with rosmarinic acid and this correlated with the level of expression of iron dependent genes. **Conclusions: the** present study showed an effective anti-leishmanial activity of rosmarinic acid and apigenin against *L. donovani* promastigotes and intracellular amastigotes and resulted in changes in the mitochondria integrity and morphology of the cells. Rosmarinic acid and apigenin induced cell cycle arrest in the G0/G1 phase. The findings suggest that rosmarinic acid could be exerting its inhibitory effect against the parasite via iron chelation which results changes in the morphology and the arrest of the cell cycle while apigenin may exert its inhibitory effects by other mechanisms.

P809/B829

Mitochondrial Pyruvate Carrier Subunits Are Required for Pyruvate-driven Respiration, Infectivity, and Intracellular Replication of *Trypanosoma Cruzi*.

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Pyruvate is the final metabolite of glycolysis. Inside the mitochondria pyruvate can be converted into acetyl-CoA, which is used as substrate for the tricarboxylic acid cycle. Pyruvate metabolism is important to balance glycolysis and oxidative phosphorylation. Pyruvate availability in mitochondria depends on its active transport by a hetero-dimer MPC1/MPC2 which is called the mitochondrial pyruvate carrier (MPC). In this work, we aimed to characterize the *Trypanosoma cruzi* MPC (TcMPC) performing gene knockout and endogenous C-terminal tagging using CRISPR/Cas9 system, and overexpression of *MPC1* and *MPC2* genes. Endogenous C-terminal tagging of *TcMPC1* and *TcMPC2* genes with 3xc-Myc showed that both proteins co-localize with MitoTracker to the mitochondria of epimastigote forms. Individual knockout of *TcMPC1* and *TcMPC2* genes was confirmed by PCR and Southern blot analyses. *TcMPC1*-KO or *TcMPC2*-KO epimastigotes did not show slower growth rate in either regular or low glucose culture medium as compared to the control cell line transfected with scrambled sgRNA. Digitonin-permeabilized *TcMPC1*-KO or *TcMPC2*-KO epimastigotes showed impaired oxygen consumption rates when pyruvate, but not succinate, was used as mitochondrial substrate as compared to the control cell line. Overexpression of *TcMPC1* or *TcMPC2* did not affect oxygen consumption rates. The results suggest that, in contrast to human MPC subunits (Nagampalli et al., 2018), they do not form homo-dimers in the absence of each other. The MPC inhibitor UK5099 (50 µM) was able to completely inhibit oxygen consumption of control cell lines using pyruvate, but not succinate, as substrate. Furthermore inhibition of succinate dehydrogenase by malonate in digitonin-permeabilized control cells impaired pyruvate-driven respiration. Ability of trypomastigotes to infect tissue-cultured cells was reduced three times and replication of intracellular amastigotes was also decreased for both *TcMPC1*-KO and *TcMPC2*-KO cell lines. In conclusion, *T. cruzi* MPC1 and MPC2 are essential for cellular respiration in the presence of pyruvate, invasion of host cells, and replication of amastigote forms. Acknowledgements: FAPESP grants 2013/50624-0 and 2017/25084-3 Key words: *Trypanosoma cruzi*, pyruvate, CRISPR/Cas9, mitochondria

P810/B830

Casein Kinase Regulates Mitochondrial Nucleoid Scission in the African Trypanosome, *Trypanosoma Brucei*.

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The mitochondrial nucleoid (kinetoplast) of the eukaryotic microbe *Trypanosoma brucei* is essential for transmission of human African trypanosomiasis from tsetse flies to humans. Little is known about mechanisms controlling division of the kinetoplast, which contains thousands of catenated circular DNA molecules (kDNA). Using RNAi and a chemical probe, we demonstrate that inhibition of casein kinase 1.2 (TbCK1.2) arrests scission of replicated kDNA, although the enzyme is not detected at the mitochondrion. We hypothesized that TbCK1.2 regulates kinetoplast division through effector proteins. We used a multi-pronged approach (quantitative phospho-proteomics, quantitative proximity biotinylation, and in *vitro* peptide kinase assays) to identify substrates that could be effectors for TbCK1.2. TbLRRP1 was de-phosphorylated after TbCK1.2 knockdown in *vivo*, and its peptide was

phosphorylated by TbCK1.2 in *vitro*. Knockdown of TbLRRP1 inhibited division of kinetoplasts, suggesting a role for the protein as an effector of TbCK1.2 signaling in *vivo.* Successful identification of an effector for TbCK1.2 suggests that our workflow may be used in other eukaryotes to study investigator-chosen biological pathways that are regulated by protein kinases.

P811/B831

Mcu Complex Physically Interacts with Mitochondrial Atp Synthasome in Trypanosomes and Mammals.

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Mitochondrial Ca^{2+} transport mediated by MCU complex plays a key role in the regulation of cell bioenergetics and life-death decisions both in trypanosomes and mammals, but the molecular mechanism has remained unclear. Here we have discovered that *Trypanosoma brucei* MCU (TbMCU), together with TbMCUb and TbMCUc, associates with the mitochondrial ATP synthase, phosphate carrier (PiC) and adenine nucleotide translocator (ANT) forming part of a potential "ATP synthasome", as detected by mass spectrometry of tandem affinity-purified TbMCU complex. Split-ubiquitin membrane-based yeast two-hybrid (MYTH) assays identified that TbMCU physically interacts with *T. brucei* ATP synthase subunit c (TbATPc). Interestingly, the direct physical MCU-ATPc interaction is conserved in trypanosomes and human cells. Combining mutagenesis analysis with MYTH assays revealed that transmembrane helices (TMHs) were determinant of the specific interactions between MCU and ATPc. In *situ* tagging, immunoprecipitation and immunofluorescence microscopy revealed that TbATPc co-immunoprecipitates with TbMCU complex (TbMCUC), and colocalizes with the TbMCUC to the mitochondria of *T. brucei.* Blue Native PAGE and immunodetection analyses indicated that the TbMCUC interacts with the ATP synthase in a large protein complex with a molecular weight of approximately 900 kDa. Ablation of the *TbMCUC* subunits by RNAi significantly increases the AMP/ATP ratio, revealing the down-regulation of ATP production in the cells. Finally, specific interaction between human MCU (HsMCU) and HsATPc was confirmed in *vitro* by mutagenesis and MYTH assays, and in *vivo* by co-immunoprecipitations. In summary, our study has identified that MCU complex physically interacts with mitochondrial ATP synthase, possibly forming an MCUC-ATP synthasome "megacomplex" that couples ADP and Pi transport with ATP synthesis, a process that is stimulated by Ca^{2+} in trypanosomes and human cells.

P812/B832

Studies Resolving Unexpected Functions and Regulation of Fructose 1,6-bisphosphatase in the Parasite *Trypanosoma Brucei*.

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Glucose-6-phosphate (G6P) is a key metabolite, required for both glycolysis and the pentose phosphate pathways. This critical compound is generated by either glycolysis or gluconeogenesis (GNG), with the former utilizing glucose while the later generates G6P from non-carbohydrate sources when glucose is scarce. In most organisms these pathways are localized together within the cytoplasm and reciprocally coordinated via allosteric regulation of key enzymes in the glycolytic and GNG pathways; phosphofructokinase (PFK) and fructose 1,6-bisphosphatase (FBPase). The protozoan parasites *Trypanosoma brucei* are unique in that most of the glycolytic and GNG pathways are localized within a specialized membrane-bound organelle called the glycosome. Additionally, in these parasites PFK and

FBPase activities are not regulated by conditions and molecules known to regulate FBPase and PFK in other systems. The coordination of the pathways is important. *T. brucei* alternates between a source of high (~5 mM) glucose in the mammalian bloodstream as the bloodstream form (BF) and the tsetse fly vector as the procyclic form (PF) where glucose is scarce. We are working to understand how glycolysis and GNG contribute to this metabolic flexibility. While FBPase protein is expressed in all stages of the lifecycle, activity has been challenging to monitor. To overcome this hurdle, we developed a sensitive in *vitro* assay and used it along with western blot analysis to follow FBPase protein and activity levels. Using these tools, we have found that FBPase protein levels were 2-fold lower in BF parasites as compared to PF, while FBPase activity was 2-fold higher. We hypothesize that the increase in activity is the result of post-translational modification(s) to the protein. In PF parasites FBPase protein and activity levels were both increased when parasites were cultured in high-glucose conditions. This observation that FBPase activity is greatest in high-glucose conditions, when glycolysis (not GNG) is expected to be the active metabolic pathway suggests that the protein may serve a function that is distinct from GNG. As we identify proteins and pathways that are affected by changes in FBPase levels, we anticipate identification of unexpected roles for FBPase in the protozoan parasite.

P813/B833

Modulation of Glucosylceramide Synthase Expression Alters the Growth and Differentiation of an an cient Protist, *Giardia Lamblia*.

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The waterborne parasite, *Giardia lamblia*, is a major cause of the zoonotic diarrheal illness (giardiasis) worldwide. *Giardia* is classified as a “model organism” to study evolution because of its compact genome and reduced metabolic pathways. It lacks emblematic eukaryotic organelles such as mitochondria and Golgi complexes. The infection by *Giardia* is transmitted by the cyst form in contaminated food, water, or feces. *Giardia* maintains a simple life cycle, i.e., trophozoites and cysts. Ingested cysts undergo excystation in the proximal small intestine, releasing trophozoites that initiate infection in the gut. Upon reaching the distal small intestine, trophozoites transform into cysts (encystation) and are excreted in stool, thus completing the life cycle. It has been shown that giardial glucosylceramide synthase/transferase (gGlcT1), an enzyme of the sphingolipid metabolic pathway, is critical for inducing encystation, cell division, and vesicular trafficking. In addition, our laboratory has reported that gGlcT1 is a dual-substrate enzyme, with separate catalytic domains that facilitate the synthesis of glucosylceramide and galactosylceramide. The goal of this project is to characterize gGlcT1 and evaluate whether the modulation of its synthesis can influence the structure of cysts since oval-shaped cysts are infective and undergo excystation. Three expression constructs were developed to express gGlcT1: (1) gGlcT1 under its own promoter with an endogenous C-terminal HA tag (endo-gGlcT1-HA); (2) an overexpression plasmid containing an α_2-tubulin promoter (α_2-Tub-gGlcT1-AU1) and (3) a plasmid under a strong giardial OCT (ornithine carbamoyltransferase) promoter (OCT-gGlcT1-HA). Under the different promoters, it was found that the enzyme expression and activity were modulated, and cyst production was reduced. Proteomic analysis showed that gGlcT1 overexpression altered the expression of glycolytic and pentose-phosphate pathways that are involved in producing energy under anaerobic conditions. Furthermore, truncation mutations of gGlcT1 provided insight into the catalytic regions important for triggering encystation and its cellular and metabolic functions. These results suggest that the regulated expression of gGlcT1 is critical for the growth and differentiation of *Giardia*, therefore it could serve as an ideal target for developing anti-giardial therapies.

P814/B834

Identification of Metazoan Transcription Factor, Which Interacts with the Minimalist Mitochondria-related Organelles of Parasitic Protist *Giardia Intestinalis.*

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Giardia intestinalis is a parasitic protist inhabiting human and animal gut. The parasite is spread via an infectious cyst, which is formed during the passage through the digestive tract of the host. *Giardia* has a very unique cellular structure. It has two nuclei, eight flagella and large adhesive disc by which it attaches to the gut epithelium. Instead of aerobic mitochondria, *Giardia* carries minimalist double-membrane organelles called mitosomes. We have identified *Giardia* homologue of Mlf1IP (Myelodysplasia-myeloid leukemia factor 1-interacting protein) in a novel cellular compartment in close proximity to the mitosomes. The protein was originally described as Metazoa-specific. However, using the large scale bioinformatic analysis, we have shown that Mlf1IP can be found in all major groups of eukaryotes. In animal cells, Mlf1IP has been characterized as a nuclear transcription factor, for which cytosolic localization has also been documented. There the protein acts as a negative regulator of cell cycle progression. We have been investigating GiMlf1IP in order to understand the functional connection between the Mlf1IP-containing compartment (Lin et al. 2019) and the mitosomes. Interestingly, various truncated forms of Mlf1IP retained its localization close to mitosomes. Using the affinity purification method, we identified mitosomal outer membrane proteins among its putative interaction partners. Surprisingly, the overexpression of Mlf1IP interferes with the encystation of the parasite, indicating its possible role in cell cycle regulation even in this single-cell eukaryote.

P815/B835

Molecular Characterization of *Giardia Lamblia* in Bivalves Collected in New York.

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Giardia lamblia, a flagellated protozoan parasite, if ingested, infects the lumen of the small intestines in humans. This parasite is found in various animals, such as dogs, cats, and birds. *G. lamblia* is most commonly transmitted to humans via ingestion of contaminated food and water. Its presence in marine environment is relatively unorthodox and its presence in public waterways is a public health concern. The goal of this study is to determine the prevalence of *G. lamblia* in Atlantic oysters *(Crassostrea virginica*) and ribbed mussels (*Geukensia demissa*) collected from Orchard Beach and Clason Point, Bronx, NY. Each year, the mussels were collected in the fall. Tissues were dissected, followed by DNA extraction and PCR analysis. Thus far, we found a marked fluctuation in the prevalence of *G. lamblia* in both bivalves from 2014-2018, ranging from no 4.5% to 42%, with no parasite detected in 2015 in the ribbed mussels collected from Orchard beach. In addition, a change in the genotype of *G. lamblia* was observed from year to year, with assemblage a being more frequent. In 2017, assemblage a was most prevalent at Orchard beach (73.68%) and sub-assemblage AI was most prevalent and Clason Point (73.46%), indicating different genotypes of *G. lamblia* at those two locations. In conclusion, Atlantic oysters and ribbed mussels may be used as a biological sentinels to detect *G. lamblia in* public waterways and reservoirs.

P816/B836

Molecular Characterization of *Giardia Lamblia* in Oysters (*Crassostrea Virginica*) Collected at Two Beach Sites in New York City in 2018.

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Giardia lamblia is a known zoonotic endoparasite that proliferates in the small intestines of vertebrates. In humans, it is responsible for giardiasis, which is an infection of the small intestines, leading to diarrhea. *G. lamblia* has also been associated with the development of irritable bowel syndrome and chronic fatigue in humans. Previous research in our laboratory and others have found a high prevalence of *G. lamblia* in tissues of bivalves living in marine environment. The objective of this research is to determine the genotype and prevalence of *Giardia lamblia* from two beach sites, less than 10 miles apart, located in the Bronx borough of New York City using molecular techniques. A total of 39 oyster specimens were collected in the fall of 2018 during low tide, 16 at Orchard Beach and 23 at Clason Point. Each specimen was dissected to isolate the digestive gland, adductor muscle, mantle, gills, and hemolymph. DNA was extracted from each tissue. To detect *G. lamblia,* nested-PCR was performed using primers that target the β-giardin gene. The prevalence of *G. lamblia* DNA was found to be 8.7% at Clason Point, while it was 12.5% at Orchard Beach, respectively. The mantle was the tissue displaying the highest prevalence of *G. lamblia* DNA. The genotype of *G. lamblia* at both locations was found to be assemblage A. In summary, variation in the prevalence of *G. lamblia* was observed between the two sites, but not in the genotype. It is important to investigate the prevalence of *G. lamblia in* oysters which are often consumed raw. In addition, oceans are commonly frequented by a large number of individuals during the hot summer months.

P817/B837

Characterization of *Giardia Lamblia in* Blue Mussels Collected from Two Beach Sites in New York City.

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Giardia lamblia is an intestinal parasite commonly found in rivers. It is the causative agent of giardiasis in humans. Bivalves have been shown to carry the intestinal parasite *G. lamblia. The* goal of this research is to determine the prevalence of *G. lamblia in* blue mussels (*Geukensia demissa*) species from both Orchard Beach and Clason Point in New York. Specimens were collected in the fall of 2018 and each specimen was dissected to harvest the mantle, gills, foot, digestive gland, abductor muscle, and hemolymph. A total of 20 specimens were tested from Clason Point and a total of 42 specimens were tested from Orchard Beach. The prevalence of *G. lamblia* for specimens collected from Clason Point was 55% and 4.80% for those collected from Orchard Beach. The *G. lamblia* parasite was found in all of the tissues except for the foot and hemolyph. Further, the mantle tissue had the highest prevalence of *G. lamblia* for Clason Point, while the gill tissue had the highest prevalence for Orchard Beach.

P818/B838

From Ciliate Biology to Physical Models of Mechanically Encoded Cell Behavior.

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Ciliates are extraordinary single celled eukaryotes that use rapid morphology changes to perform fast animal-like behaviors such as jumping, grabbing and hunting. These behaviors are built from a toolbox of molecular components—motile cilia, cytoskeletal structures, and contractile machinery—that are

regulated in space through different cell geometries and in time through signaling controllers that rapidly regulate activity. To understand how these activities can be used to build microscale molecular machines with complex behaviors, we analyzed the hunting dynamics of the predatory ciliate *Lacrymaria olor*, which locates and captures prey using the tip of a slender "neck" that can rapidly extend more than ten times its body length (500 µm from its body) and retract in seconds. Although its hunting behavior appears complex, a detailed quantitative analysis of the cell's shape dynamics shows that it actually locates prey through rapid, near-random, dense sampling of the area within the neck's radius. To execute random search in a challenging low-Reynolds number environment, the cell exploits elasto-hydrodynamic buckling responses of its cytoskeleton to repeated cycles of extension and retraction using its ciliary and contractile machinery. Inspired by these observations, we develop a high fidelity elasto-hydrodynamic physical model in which arbitrary cytoskeletal geometries can be coupled to a surface activity to generate emergent dynamics. Using this model we first explore the cell's hunting behavior by elucidating the design principles and control signals to the underlying molecular components that underlie this behavior. More broadly, we explore how such a model can be used to explain the diversity of forms and functions within the *ciliaphora*.

P819/B839

The Fine Structure of the Tentacular Apparatus of Adult Ctenophore*Mnemiopsis Leidyi*.

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The common Western Atlantic ctenophore *Mnemiopsis leidyi* is currently the object of intense ecological and physiological study because several multigenetic molecular analyses have shown Phylum Ctenophora to be the sister taxon to all other animals (Science 342:1242592; Nature 510:109). The tentacular apparatus of ctenophores is critical to feeding and very likely bears a diversity of sensory functions. Previous studies of the cydippid *Pleurobrachia pileus* suggest that the tentacular bulb is an integrative center for afferent signals arising from the distal tentacle (Trans Roy Soc. B 339:1) as well as a cell proliferation zone (Dev Biol 350:183). The tentacular apparatus of adult cydippid and lobate ctenophores have previously been presumed to be superficially similar but as we show here, display distinct cellular organization. Here, we show a new view of tentacular apparatus structure and compare to the original work of Mayer in 1912. Oblique illumination light microscopy reveals a fan-like ridge of tissue located on the center of the bulb previously misinterpreted to be the 'principle tentacle' (Hyman, 1940). The fan comprises hundreds of tentillae that continually arise from the center edge of the ridge, thus each tentillum connects directly to the bulb. These findings are contrary to previous conclusions that the tentillae are side branches of a larger cydippid-like tentacle embedded in the epithelia of the food groove. We created a lesion in the tentacular groove to attempt to establish continuity of the tentillae within the food groove. We used time-lapse recording to reveal the post-incision recovery mechanisms of the tentacular groove and the tentillae. Tentillae do not arise locally from the tentacular groove but instead form at the bulb and extend the full length of the feeding apparatus. Thin section analysis of the tentacular bulb reveals distinct regions in the bulb. Major features include: 1) that the bulb emerges from the epithelium at its oralmost end; 2) a pair of canals that underlie the bulb along its full length; 3) a distinct region of bulb myo-neuroepithelium is located on the mid-line in the fan; 4) a region of tentillar longitudinal fission and growth (TLFG) that moves laterally to either side of the bulb to 'feed' tentillae within the feeding groove; 5) a region of apparent cellular proliferation specifically underlying the TLFG; 6) cells with highly condensed chromatin aggregates, that we interpret to be indicative of an unusual form of cellular suicide.

P820/B840

A *Plasmodium* Homolog of Er Tubule-forming Proteins Is Required for Parasite Virulence.

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Reticulon and REEP family of proteins stabilize the high curvature of endoplasmic reticulum (ER) tubules. *Plasmodium berghei* Yop1 (*Pb*Yop1) is a REEP5 homolog in the *Plasmodium* genome. Here we characterize its function using a gene-knockout (*Pb*yop1Δ). *Pb*yop1Δ parasites display loss of ER architecture, an enlarged digestive vacuole and severe attenuation of the asexual cycle. Sporozoites have reduced speed and are slower to invade cultured hepatocytes. We propose that *Pb*YOP1's disruption leads to defects in trafficking and secretion of a subset of proteins required for parasite development and invasion of host cells. Consistent with this defect, *Pb*yop1Δ-infected mice display lower incidence of experimental cerebral malaria (ECM) and reduced brain expression of CD8+ T-cell effectors. ICAM-1 protein expression is specifically reduced in the brainstem, indicating decreased activation of endothelial cells, which are known to cross-present parasite antigens to CD8+ T-cells. We propose that *Pb*YOP1-dependent processes are required for parasite-mediated activation of CD8+ T-cells in the brain during ECM.

P821/B841

Elucidating the Origin of an imal Cell Types through the Mechanisms of Cell Type Regulation in the Choanoflagellate *Salpingoeca Rosetta*.

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In every animal, distinct cell types perform specialized roles, yet we have little insight into the evolutionary trajectory that led from unicellular life to this incredible collaboration of specialized cells. While animals specify cell types using interconnected, hierarchical, and modular transcriptional networks, it is poorly understood how the nature and evolution of these networks influenced the origin of animal cell types. One barrier to this understanding is the limited phylogenetic diversity of taxa in which the transcriptional regulation of cell types has been studied. I am unraveling the transcriptional control of a cell type transition in the choanoflagellate Salpingoeca rosetta, one of the closest living relatives of animals. I am also assessing the transcriptional modularity of sub-cellular functions in *S. rosetta*, the patterning of cis-regulatory information across its condensed genome, and the role of histone post-translational modifications with regards to cell type regulation and transcriptional control. This study will broaden our understanding of cell type regulation across the phylogenetic tree, particularly with detailed information about the closest living relatives of animals. Additionally, this knowledge may help to infer attributes of pre-animal transcriptional networks and guide our understanding of the origin of animal cellular differentiation.

P822/B842

Leukocyte Transendothelial Migration Mimicry:a Novel Mechanism In*Trypanosoma Cruzi*-host Interaction.

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The Chagas disease parasite *Trypanosoma cruzi* primarily infects the heart and gastrointestinal tract and the infection may trigger structural and functional aberrations such as cardiomegaly, megaesophagus, and megacolon. To reach the organ parenchyma, where it thrives and multiplies inside a variety of cell types, *T. cruzi* must cross the vascular endothelium. If it does not, the protozoan pathogen will likely perish in the bloodstream**.** However, the mechanisms underlying *T. cruzi*transendothelial migration (TEM) remain to be determined. We hypothesize that *T. cruzi* takes advantage of the molecular machinery leukocytes use for TEM, a pathway essential for immune cells to access tissues to combat injury and microbial invaders. Much is known about the molecular interactions required for multistep leukocyte TEM. The first step consists of leukocytes decelerating by binding to the adhesion molecules P-selectin (SELP) and E- selectin (SELE) located on the endothelial luminal surface. The binding requires P- and E- selectins reaction with sialyl and sulfate residues on the ligand P-Selectin Glycoprotein Ligand 1 (PSGL-1) expressed by leukocytes. Our results show that endothelial cells exposed to *T. cruzi* strongly upregulate both SELP and SELE genes, suggesting that *T. cruzi* controls expression of at least part of the molecules responsible for TEM. Both SELP and SELE bind to *T. cruzi* in a dose-dependent and saturable manner, and the binding requires sialyl and sulfate residues, akin to leukocyte-PSGL-1 recognition. Furthermore, preincubation of *T. cruzi*with SELP or SELE prevents *T. cruzi* entry into endothelial cells, implying that *T. cruzi* interaction with selectins guide migration of the parasite through the endothelium. Thus, our findings suggest that *T. cruzi* mimics leukocytes in traversing the endothelial barrier in its way to reach parenchymal host cells. Aside from unveiling a novel mechanism in *T. cruzi*-host interaction, understanding *T. cruzi* TEM may also suggest a novel therapeutic avenue for incurable Chagas disease.

P823/B843

Demonstration of Rna Interference by Feeding In*Stentor Polymorphus*, a Potential Model for the Study of Host-symbiont Interactions.

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Stentor is a genus of large trumpet-shaped unicellular organisms in the ciliate phylum. Classically they have been used as models for morphogenesis due to their large size and ability to regenerate, but some *Stentor* species have features that make them useful models for other types of studies as well. *Stentor polymorphus* is a widely distributed species that harbors green algal endosymbionts from the *Chlorella* genus. While interesting phenomenology in this species has been described, molecular tools have never been developed in this system. As technology has advanced, the use of emerging models like *S. polymorphus* has become more prevalent, and recently a set of transcriptomes for *S. polymorphus* was published. However, there are still technical hurdles to using this emerging model as an experimental system in the lab. Here I describe the identification and culture of a *S. polymorphus* population from North Carolina as well as the identification and cloning of homologs of α-tubulin and *MOB-1*. Additionally, I demonstrate that RNA interference (RNAi) by feeding is effective against both of these homologs in *S. polymorphus*. These two RNAi targets were previously validated in *S. coeruleus and* the observed phenotypes were similar to the phenotypes observed in *S. polymorphus*. A direct comparison between the two species revealed that RNAi appeared to be less effective in *S. polymorphus*. The ability

to perform RNAi in *S. polymorphus* strengthens its use as an emerging model for fascinating biology like the mechanisms of unicellular regeneration or host-symbiont interactions and suggests that RNAi by feeding might be more broadly effective across the *Stentor* genus.

P824/B844

Single Domain Antibodies Targeting Histones as a New Tool for Detection and Discrimination between *Leishmania (v.) Braziliensis*And *Leishmania (v.) Peruviana*.

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American cutaneous leishmaniasis (ACL) is a major public health parasitic disease in New World. Most endemic areas lack of an efficient point of care diagnosis test and solely have access to microscope for giemsa-stain smear diagnosis depending in parasites number in a wound. Drug treatment options should be decided by *Leishmania* species essentially when a severe form caused by *Leishmania (V.) braziliensis* is the cause agent. Molecular tools are costly and need requirements restricted for point of care centers. We decided to use single domain antibodies for their characteristic of small-sized, heat stability, high solubility, antigen specificity and reproducible production. In the present study, our aim is to determinate single domain antibodies for Leishmania detection and identification. We incubated the single domain antibody phage library, PREDATOR, against native histones of *L. (V.) braziliensis* promastigotes by a stringent process with positive and negative selections based on Phage Display technology. The single domain antibody S4B1 was able to detect *L. (V.) braziliensis* and *L. (V.) peruviana* unlike others species. Using blot assays we are able to discriminated these two closest species using a conserved protein like histones. In ELISA assays, S4B1 showed a limit detection less than 630 ng/ml of purified native histones. In addition, when we evaluated antibody S4B1 by immunohistochemistry assays, we were able to detect the nucleus of amastigote, stage present in naturally infected human biopsy. In conclusion, a biopanning process of the PREDATOR library allowed us to select the antibody S4B1, particular for high sensitivity and very specific detection capability. This antibody S4B1 is in developing for conditions for be using in point of care centers.

P825/B845

Tglaforin, a Glucan Phosphatase, Modulates Central Carbon Metabolism in *Toxoplasma Gondii*.

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Toxoplasma gondii is an intracellular, protozoan parasite that currently infects roughly one-third of humans worldwide. The asexual stage *of T. gondii*, found in humans, consists of two morphologically distinct forms: the tachyzoite and the bradyzoite. The rapidly dividing tachyzoites define the acute phase of infection, and convert to slow growing, encysted bradyzoites during the chronic phase. A distinguishing characteristic of bradyzoites, noticeably absent in tachyzoites, is their ability to accumulate starch-like glucose polymers known as amylopectin granules (AGs). Importantly, the *T. gondii* genome encodes the activities needed for AG (starch) utilization. These include a glucan-water dikinase (TgGWD; TGME49_214260), a glucan phosphatase (TgLaforin TGME49_205290), and multiple amylases. These activities comprise a cycle of reversible phosphorylation required for starch degradation. To determine if such a cycle is critical for AG degradation in *T. gondii*, we utilized both biochemical and molecular genetics approaches. We expressed active, recombinant TgLaforin, which

necessitated the use of the Sf9 insect cell expression system. Using several biochemical assays, we confirmed that TgLaforin is an active glucan phosphatase. Biophysical techniques including multi-angle light scattering (MALS) and differential scanning fluorimetry (DSF) have revealed an oligomeric state for TgLaforin, and provided insights into protein organization, stability, and substrate specificities. A CRISPR-Cas9 knockout of TgLaforin in tachyzoites results in a minimal fitness defects in nutrient-replete media. However, potential compensatory changes in the TgLaforin-KO suggest a rewiring of central carbon metabolism, as revealed by metabolomics analyses. Unexpectedly, these findings were confirmed by the KO's dependence on glutamine for growth, even in glucose replete conditions. Moreover, a tachyzoite to bradyzoite conversion assay using alkaline stress revealed that KOs retain tachyzoite markers despite the simultaneous development of bradyzoite characteristics. Even though tachyzoites are not known to utilize AGs, these results suggest that AG metabolism may play a role throughout the asexual stage of these parasites.

P826/B846

***Trichomonas Vaginalis* Infection Causes Pro-neoplastic Changes in Prostate Epithelial Cells & in Orthotopic Rat Model.**

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Recent studies suggest that Trichomonosis, caused by the human-infective parasite *Trichomonas vaginalis* (Tv), is associated with increased risk of prostate cancer. However, the detailed mechanism of Tv infection associated inflammation and the generation of anomalies (premalignant/ malignant) in prostate is not clear due to the lack of an acceptable experimental model. Hence, host-parasite studies in *vitro* and development of a rodent model could be important in improving our understanding of the prostatic anomalies caused by parasite. We hypothesized that the effect of Tv infection on multiple inflammatory mediators in the prostate could contribute to the development of pathological changes. A co-culture of RWPE-1 cells with Tv modulated multiple pro-inflammatory cytokines/chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-6, IL-7,IL-12, IL-16, PDGF, IFN-γ, TGF-β1, GC-SF, GM-CSF, sTNFR1, sTNFR2, IL-8, MIP-1α, I-309, MCP-2, EOTAXIN, MIG, and RANTES), various other critical inflammatory mediators (STAT-3, NF-κB p65, COX-2, MIF) and enhanced cell survival pathways (including AKT, ERK, p38). In addition, prostate cancer cell line (DU145) underwent a phenotypic shift from epithelial to a mesenchymal-like phenotype when co-cultured with Tv. The acquired phenotype involved the enhancement of cell migration and invasion, with an increased expression of vimentin, FAK and decreased expression of E-cadherin. Furthermore, a Tv infected Sprague Dawley rat model was developed by intra-prostatic injection of Tv and harvesting of prostate glands at different time intervals (10, 30, 50, 70, 90, 110 and 130 days) for experiments. Histology and Immunohistochemistry data established that the parasite infection promoted cell proliferation of the host prostate cells by increased expression of Ki-67 and PCNA. Consistent with our in *vitro* results, we found that Tv infection altered multiple pro-inflammatory signalling proteins and cytokines/chemokines in the host prostatic cells. In conformity with the co-culture experiments, we also observed high expression of PSA (a biomarker of Prostate Cancer) in Tv-infected rat prostate that directly correlated with the severity of Tv infection. Interestingly, Tv induced prostatic anomalies could be reversed in the rat model by administration of metronidazole (USFDA approved 5-nitroimadazole anti-trichomonal drug). Overall, we have developed a Tv-infected prostate model in rat with an aim to delineate the pathogenic mechanisms associated with the parasite infection in humans. This model offers critical insights about the complex interactions between the parasite and

host, which may be useful in the prevention and/or development of intervention strategies against the parasite-induced prostatic neoplasia.

P827/B847

Gain-of-function Mutation in Erythrocyte Mechanoreceptor Piezo1 Causes Dense Cells Resistant to Malaria Parasite Invasion.

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PIEZO1 is a non-selective cation channel in mammalian, but not parasite cells, that is activated by mechanical stimulation. In erythrocytes, PIEZO1 functions in tandem with $K_{Ca}3.1$ (Ca^{2+}-dependent K^+ or Gardos channel) to controls cell volume. In mouse models and in humans, gain of function mutations of host mechanoreceptor PIEZO1 (GoF-PIEZO1) led to impaired malaria parasite propagation in erythrocytes. GoF mutations of PIEZO1 are linked to hereditary xerocytosis, a human hematological disorder featuring dehydrated erythrocytes probably due to increased intracellular Ca^{2+} and decreased intracellular K^+. Indeed, dehydration of sickle cells correlates with protection against severe malaria in sickle carriers. Blood of three out of 22 tested African American donors had the most prevalent GoF-PIEZO1 mutation (E756del). Comprehensive analysis of *P. falciparum* replication in erythrocytes from these E756del carriers revealed no major defects. However, some schizonts grown in carriers' blood had distorted or dehydrated morphology, reminiscent of schizonts we previously analyzed in sickle cells from AS carriers. Isolated, dense erythrocytes from E756del carriers showed impaired parasite replication. Interestingly, isolated dense erythrocytes from non-GoF subjects showed impaired replication as well. The parasite erythrocyte cycle in dense cells had normal duration, kinetics of egress, and numbers of de-novo produced parasites per schizont, but impaired parasite invasion. Chemical activation of the PIEZO1 channel in normal erythrocytes by PIEZO1 agonist Yoda1 caused their dose-dependent dehydration, persisting at least three hours after its washout. Morphological abnormalities persisted even longer. Replication of parasites in Yoda-treated erythrocytes was slightly diminished in whole blood and severely diminished in Yoda-treated dense erythrocytes. In summary, dense erythrocytes, whether from GoF-PIEZO1 E756del carriers or not, are defective in supporting *P. falciparum* replication in *vitro*. Dehydration to the point of densification may be a final common pathway to understanding the mechanism of invasion in malaria parasite biology.

Fungi

P828/B848

Controlling the Developmental Switch between a Human-like to a Yeast-like Cell in a Chytrid Fungus.

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Actin is a core and highly conserved component in every eukaryotic lineage. Despite this conservation, actin networks drive a vast array of behaviors, often within the same cell. Our previous work shows that the pathogenic chytrid fungus *Batrachochytrium dendrobatidis* (Bd) exhibits temporally and phenotypically distinct actin networks. During its juvenile stage, Bd uses a single posterior flagellum to swim through water and actin-filled pseudopods and an actin cortex to crawl across surfaces, resembling amoeboid animal cells. As the chytrid matures, Bd switches to a sessile stage utilizing actin

patches and cables to facilitate the growth of a hyphal-like rhizoid and an exit tube for the internally replicated juveniles, resembling filamentous fungal lineages. We discovered that mucin, a signature of Bd's host environment, acts as an external switch to trigger the transition between the two life stages within minutes. Further, we have employed small molecule inhibitors of actin assembly to impair actin pseudopods and filopodia-like protrusions prior to this transition. With these tools, we can control both Bd's transition and ability to assemble distinct actin networks, to ultimately characterize the role of actin during development. Moreover, we are currently determining the mechanisms of Bd's reaction to mucin by assessing its substrate trigger specificity, its characteristic cell polarity markers, and potential developmental pathways through drug inhibition. In addition to answering questions about actin evolution and network regulation, a better understanding of Bd's cell biology will aid in our efforts to combat chytridiomycosis - skin infections causing a dramatic decline in amphibian populations worldwide.

P829/B849

A New Window into the Evolution of Fungal and an imal Cell Biology: Genetic Transformation of the Chytrid *Spizellomyces*.

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Chytrids are deep fungal lineages that display traits associated with both animals and fungi, making them evolutionary transitional lineages. Chytrid spores and gametes, called zoospores, lack a cell wall, move with amoeboid movement and swim with a motile cilium nucleated from a centriole, all characters found in animal cells but lost in other fungi. The zoosporic stage then transitions into a more typical fungal developmental program, by building a cell wall and undergoing synchronous nuclear divisions without cytokinesis before cellularizing and releasing new zoospores. The later stages of this developmental program are reminiscent of the cellularization of the *Drosophila* embryo and *Dictyostellium* polarized epithelial development, suggesting that the toolkit for multicellularity may have predated the divergence of the animal and fungal lineage. This unusual cell biology makes chytrids important research organisms to explore the emergence of multicellularity. Moreover, chytrids can provide an evolutionary framework for integrating animal and yeast cell biology: this single research organism developmentally switches from a ciliated amoeba with an actin cortex and protrusions into a "yeast" that undergoes a multicellular-like developmental program. To capitalize on chytrid biology, we have developed a method for stable and reliable genetic transformation of the soil chytrid *Spizellomyces*. By combining expression of fluorescently tagged proteins and live-cell imaging we have taken the first steps towards developing *Spizellomyces* as a model for animal-fungi evolutionary cell biology. We show how polymerized actin drives zoospore motility, and forms transient perinuclear actin shells that coincide with nuclear division. Additionally, we describe how the formation of membrane cleavage planes during cellularization may depend on the formation of three-dimensional polygonal territories of polymerized actin. We are currently extending these tools to use *Spizellomyces* as a model system to study the evolution of key animal and fungal traits, particularly cell cycle regulation, the evolution of cellular motility and the fungal developmental program.

P830/B850

Quantifying the Spatio:temporal Levels of Reactive Oxygen Species in *Cochliobolus Heterostrophus* during Germination and Interaction with Maize Using the HyPer Sensor.

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The interplay between plant host and fungal pathogens during infection is a complex process that is in part defined by regulation of Reactive Oxygen Species (ROS). It is well established that plants use ROS as a key component of innate immunity, growth and development. An alogously, fungi need ROS for development and production of necessary infection structures. In both organisms, ROS production must be carefully regulated to prevent deleterious effects. Our research is exploring the ROS kinetics in the necrotrophic ascomycete *Cochliobolus heterostrophus* which infects maize. To aid in the understanding of this process we have transformed *C. heterostrophus to* express a genetically encoded fluorescent reporter for hydrogen peroxide (H_2O_2) called HyPer. Using confocal microscopy, we are employing an in vitro and in planta analysis for a complete understanding of the role of ROS within *C. heterostrophus*. The in vitro analysis is quantifying the response of conidia to external stimuli in a gravity perfusion system on a confocal microscope. The perfusion analysis permits us to flow oxidative (H_2O_2) , reducing (dithiothreitol), or plant extracts across the conidia in a controlled manner and visualize the kinetics of the response. The second approach is imaging *C. heterostrophu*s ROS levels during infection of B73, a susceptible maize line. This is a well studied line and commonly used to generate hybrids. Single conidium are image from germination to appressorial development and entrance into the host and the fungal ROS levels are quantified. During infection there is a decrease in ROS seen as the invasive hyphae enters the host, after which it increases back to pre-infection levels. The future quantification of the HyPer sensor during infection of resistant lines will provide insight into the role ROS plays in a necrotrophic pathogenic interaction and provide useful information in breeding more robust maize lines.

P831/B851

Antifungal Mechanism of Curcumin in *Candida Albicans* Mediated by Nitric Oxide.

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One of the most pathogenic fungi existing, *Candida albicans* have caused great threats to humans. In an objective to discover antifungal effect regarding *C*. A*lbicans*, we conducted study on curcumin, a major bioactive compound. Using DAF-FM Diacetate dye, it has been shown that curcumin induced increased nitric oxide generation in *C*. A*lbicans*. Increase in nitric oxide generation was followed by increase in mitochondrial reactive oxygen species (ROS) and was monitored using MitoSOX Red dye. Mitochondrial calcium uptake was also monitored using Rhod-2AM and mitochondrial membrane potential decreased compared to untreated ones when cells were treated with curcumin using JC-1 dye. Moreover, curcumin-treated cells showed increased fluorescence intensity when using MitoTracker Green, which indicates that mitochondrial mass increased after curcumin treatment. Results show that mitochondria in *C*. A*lbicans* do not function normally after treatment of curcumin. Additionally, curcumin displayed membrane depolarization when using $DiBAC_4(3)$. In summary, mitochondrial dysfunction and membrane depolarization are induced after curcumin treatment, contributing in antifungal mechanism in *C*. A*lbicans*. When conducting study, L-NG-Nitroarginine Methyl Ester (L-NAME), which is a NOS inhibitor, was applied in every experiment. In all experiment, L-NAME pre-treated cells blocked the effects

induced by curcumin, which indicates that presence of nitric oxide affects the overall mechanism. In conclusion, curcumin displays antifungal activity in *Candida albicans* mediated by nitric oxide.

P832/B852

Localization of *frequency* mRNA in Biomolecular Condensates Contributes to Period Length Determination in the *Neurospora crassa* Circadian Clock.

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Circadian rhythms in animals and fungi are generated by a transcription - translation negative feedback loop. In *Neurospora crassa,* the WCC, a complex of WHITE COLLAR - 1 (WC-1) and WC-2, is the major transcription factor on the positive arm of the clock, while a multi-protein complex nucleated by FREQUENCY (FRQ) acts on WCC to repress its function. This feedback loop requires approximately 24-hours for a complete cycle, and has been extensively studied at the tissue, organismal, and population level for several decades using biochemical and genetic techniques. Spatiotemporal dynamics of core clock molecules at the cellular level, however, have remained elusive. In particular we are interested in the mechanism underlying the approximately 4 hour delay between peaks of *frq* mRNA accumulation and expression of FRQ protein, a delay that contributes to the long 24 hr circadian period length. An additional spur arose from recent extensive circadian transcriptomics and proteomics data that revealed unexpectedly large temporal delays between transcription and translation: a quarter of the proteome is circadianly regulated with a median delay between RNA peak and protein peak of ~10 hrs. We became curious as to how mRNA could be protected from degradation long enough to facilitate these temporal delays. We have identified a novel RNA binding protein that undergoes liquid-liquid phase separation, interacts with *frq* mRNA, and plays a role in the observed perinuclear enrichment of *frq* mRNA*. The* RNA-binding protein shows heterogeneous distribution throughout the cytoplasm and displays dynamic liquid like behavior in live-cell imaging. In knockouts lacking the protein, the heterogeneous perinuclear localization of the *frq* transcript is lost and a significant lengthening of the circadian period is observed. We hypothesize that sequestration of clock related transcripts through phase separation could facilitate local translation and/or protection of transcripts. Additionally, sequestration of circadian mRNA in biomolecular condensates could contribute to temporal delays between mRNA expression and protein accumulation, subsequently affecting the overall temporal mechanism of the clock.

P833/B853

A Polyclonal Sera Against Trimethyl Chitosan Nanoparticles Facilitates Detection of Phytopathogenic Fungi.

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Our study aims to provoke tools for the first-line detection of fungal infection in plants or any other fungal contamination in various human consumables. Chitin is one of the most abundant fungal cell wall polysaccharide that's naturally deacetylated to chitosan when the pathogenic fungi infect the host. During infection, this deacetylation process serves to represent the pathogenicity mechanisms to protect the fungi from plant extracellular chitinases because chitosan is a poor substrate for chitinases. This process ramified in the fungal cell wall modulation and plant-pathogen communication. Thusly, detection of chitosan could potentially aid in the detection of fungal contamination. Previous studies have reported that chitosan is a poor immunogen hence, we used trimethyl chitosan nanoparticles

(TMC) as an antigen to enhance the immunogenicity. We used several phytopathogenic fungi strains for the study. Mice were immunized with TMC nanoparticles to generate polyclonal sera. This sera could provide an enhanced humoral immune response and generate a rich & heterogeneous repertoire of antibodies against chitosan & TMC. The binding affinity of the sera with fungal cell wall was analyzed by various techniques such as ELISA, langmuir isotherm, confocal microscopy & ITC (Isothermal Calorimetry) and it was found that the polyclonal sera were able to detect chitosan in the fungal cell wall. Interestingly, it was found that the detection specificity varied among the strains in proportion to the chitin content of their cell wall. In ELISA, *Fusarium oxysporium* was detected with the highest affinity while *Trichoderma ressei* was detected with the least affinity. To ensure specificity and high binding capacity we performed adsorption isotherm & ITC, additionally confirmed by confocal microscopy. The notion of generating this antibody repertoire against chitosan, utilizing a methylated derivative of chitosan as nanoparticles is a novel attempt in the direction of development of detection tools for fungal contamination. The polyclonal antibody repertoire generated & experimentally verified in this study can be applied in quality control assays, in infection detection assays and as a tool to analyze the structure of fungal cell walls. Conclusively, this study could prove to be beneficial in the detection of human fungal pathogens involved in the different fungal infections.

P834/B854

Inhibition of Hsp90 Regulates Fluconazole Resistance in the Pathogenic Fungus *Cryptococcus Neoformans*.

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Cryptococcus neoformans is an encapsulated yeast, found in the lungs of immunocompromised hosts. In the human host, *Cryptococcus* is subject to multiple forms of environmental stress, including heat stress. Heat shock protein 90 (Hsp90) is an essential protein that has been shown to protect fungal cells against extreme temperature in the human host and to assist in proper cell division. Hsp90 has also been implicated in resistance to the anti-fungal drug fluconazole. To better characterize the role of Hsp90 in fluconazole resistance in *Cryptococcus* we addressed two questions: 1. Does concomitant partial inhibition of Hsp90 and treatment with fluconazole lead to increased sensitivity to fluconazole and 2. Does prolonged partial inhibition of Hsp90 prior to treatment with fluconazole lead to subsequent increase in development of fluconazole resistance. Using growth assays, disk assays, and a standardized e-test to determine the Minimum Inhibitory Concentration (MIC) of fluconazole, we found that indeed, pharmacological partial inhibition of Hsp90 with radicicol, concomitant with fluconazole treatment leads to increased sensitivity to fluconazole. However, initial pre-treatment of *Cryptococcus* with sub-inhibitory concentration of radicicol followed by exposure to fluconazole leads to development of fluconazole resistant *Cryptococcus* colonies. We characterized the resistant colonies, which show a lower MIC to fluconazole compared to cells that were never exposed to radicicol and fluconazole. Cells derived from the resistant colonies exhibit slower growth on drug-free media, and lose resistance to the drug after passaging in drug-free media. Based on the fact that resistance to fluconazole is lost when fluconazole is removed from the environment, we infer that compromising Hsp90 function during the pre-treatment with radicicol triggers aneuploidy in *Cryptococcus neoformans*. Furthermore, we speculate that the resistant colonies are aneuploids that possess an altered chromosome number to maintain drug resistance in response to an environmental insult, such as presence of fluconazole.

P835/B855

Investigating How the Septin Cytoskeleton Controls Morphogenesis in Marine Fungi.

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Complex cell morphologies are attributed to the dynamics and plasticity of the cytoskeleton. An understudied component of this framework is the septin cytoskeleton. Septins are filament-forming, GTP-binding proteins important for coordination of cell cycle progression, actin and microtubule organization, polarized cell growth, and membrane remodeling. Septins were discovered and have been most intensively studied in the budding yeast, *Saccharomyces cerevisiae*. In late G1 of budding yeast, septins form a cortical ring structure near the bud site, and at the time of bud emergence, the ring expands into a rigid septin collar, spanning the whole bud neck and scaffolds other cytokinetic factors. Despite their conserved role in the eukaryotic cell cycle, many aspects of septin assembly and function in cell morphology remain mysterious. To study the biophysical properties of septins in the context of variable cell morphogenesis, we are using a set of species of black yeasts isolated from marine environments surrounding Woods Hole, MA. The marine environment presents a variety of stresses for fungi including high osmotic and oligotrophic conditions, UV exposure, temperature fluctuations and limited substrates that necessitate particular morphological adaptations. To assess morphogenesis and division patterns at the single cell level, we filmed growth using high-magnification, differential interference contrast (DIC) time-lapse microscopy. Our single-cell analyses revealed remarkably distinct morphologies from the conventional model yeasts including multiple simultaneous budding events, division through a combination of budding and fission, and consecutive orthogonal septations. We hypothesize that some of these unique patterns of morphogenesis are linked to intrinsic features of septin polymerization and interactions of septins with downstream effectors. Bioinformatic comparisons of septin sequences show regions of divergence from the conventional yeast models *S. cerevisiae* and *S. pombe* that are conserved in the black yeast species. Many of these residue changes are located within key structural elements, such as the GTPase domain and C-terminal coiled-coil tail. This is interesting as septin oligomeric structures assemble via interactions between these domains; changes in these interactions could influence the biophysical properties of these proteins such as flexibility, annealing, fragmentation, and bundling, which could in turn alter their cellular function. Using comparative analysis of the genome, cell biology and biophysics of septins from these fungi, we can learn how the plasticity the septin cytoskeleton can generate different cell morphologies.

P836/B856

Role of Heat and Oxidative Stress on Sensitivity to Anti-fungal Drugs in the Pathogenic Fungus Cryptococcus Neoformans.

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Role of heat and oxidative stress on sensitivity to anti-fungal drugs in the pathogenic fungus *Cryptococcus neoformans*

Emily Lupinacci[1], Tyler Carlson[1], Katie Mosely[1], Corbyn Pomykata[1] and Srikripa Chandrasekaran[1] [1]Department of Biology, Furman University, Greenville, SC *Cryptococcus neoformans* is an opportunistic pathogen, infecting the lungs of immunocompromised patients, which can further lead to cryptococcal meningitis. The fungistatic drug, fluconazole (FLC), and the fungicidal drug, Amphotericin B (AmpB), are used to treat cryptococcal infections. While FLC inhibits cells by lowering the production of ergosterol, AmpB kills the cells by binding to ergosterol and permeabilizing

plasma membrane. AmpB and to a lesser extent also FLC have been implicated in generating increased levels of Reactive Oxygen Species (ROS), thereby contributing to the inhibition of *C. neoformans*. We wanted to understand how the sensitivity of *C. neoformans* to FLC and AmpB is influenced in the presence of oxidative, heat and nutrient stress. We grew *C. neoformans* in rich media such as YPD (Yeast Peptone Dextrose broth) or poor media such as YNB (Yeast Nitrogen base) in the presence and absence of various antioxidants and at varying temperatures. We found increased sensitivity to FLC at increasing temperatures but no effect of high temperature on the sensitivity of *C. neoformans* to Amp B. The antioxidants RA, AA, and PDTC, but not GSH, could rescue *C. neoformans* from FLC sensitivity, while only GSH (not RA, AA, PDTC) could rescue *C. neoformans* from sensitivity to AmpB. *C. neoformans* showed increased sensitivity to FLC and moderate sensitivity to AmpB in rich medium (YPD), while under poor nutrient conditions (YNB), *C. neoformans* showed higher sensitivity to AmpB and decreased sensitivity to FLC. Our results indicate that sensitivity of *C. neoformans* to FLC v/s AmpB varies under different environmental conditions. This difference in sensitivity could be due to contrasting role ergosterol plays in the molecular responses elicited by *C. neoformans* in the presence of either AmpB or FLC.

Defining Therapeutic Targets and New Therapeutics 1

P837/B858

Retina Specific Laminins Model Interphotoreceptor Matrix to Drive Differentiation of Photoreceptors from Human Pluripotent Stem Cells.

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Visual cycle is dependent on the functional interplay between retinal pigmented epithelium (RPE) and photoreceptors. Therefore, photoreceptors offer promising role in retinal cell therapy for treatment of retinal diseases that involve loss of these cells such as Aged Macular Degeneration (AMD). The light sensitive photoreceptors are anchored on the monolayer RPE with the support of specific laminin isoforms. At least 16 laminin isoforms (LN) present in the human extracellular matrix, play specific roles on diverse cellular differentiation and phenotype stability. Most recently, LN-221 and LN-511 have been demonstrated for robust hESC- cardiomyocyte differentiation and expansion of human epidermal keratinocytes respectively. We currently show that the inter-photoreceptor matrix could be modeled by using retina specific laminin isoforms; LN-523 and LN-521. This mimetic retina matrix like surface has shown to promote the differentiation of hESCs into photoreceptor progenitors in a simplified two steps protocol. Our single cell transcriptome analysis shows that the photoreceptor markers including rod and cone precursors; CRX, RCVRN, NRL and Pde6H are expressed in the differentiated photoreceptors as early as Day 30. The specific differentiation time points recapitulate the step wise in *vivo* retinogenesis. Since this approach is not dependent on the formation of retinal organoids and devoid of manual excision and re-plating, the reproducibility between retinal differentiation batches is very high. The functional role of the engrafted hESC- derived photoreceptor progenitors in the post-transplanted eyes after 1 month show synaptic connectivity into the rabbit host tissue. In conclusion, this retinal organoid free method robustly drives the hESC differentiation to the photoreceptor progenitor lineage, suggesting that retina specific laminin play functional role during early human retinal development. Most importantly, we have shown the feasibility and efficacy of using these laminin based differentiated cells for transplantation in the large eyed pre-clinical model. This xenogen free and chemically defined

method may constitute an important step towards clinically safe and functional hESC- derived photoreceptors to treat vision loss.

P838/B859

Quantitative Surface Proteomics in Retinal Pigment Epithelium Reveals Novel Cross-talk Pathway Inhibitory of Choroidal an giogenesis.

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Background: the retinal pigment epithelium (RPE) is polarized into apical and basolateral plasma membrane domains, which interact with photoreceptors and the underlying choroidal blood vessels respectively. Dysfunction of the RPE causes photoreceptor degeneration during age-related macular degeneration (AMD), the leading cause of blindness in the elderly. There is a need to develop treatments for AMD since most cases cannot be cured. Only a subtype, “wet” AMD, can be treated by blocking vascular endothelium growth factor (VEGF), which is produced in excess by the RPE and causes choroidal blood vessels to proliferate and leak. However, anti-VEGF treatments can develop resistance and cause adverse side effects. Objective: to identify cross-talk pathways between the RPE and choroid that could provide targets for more effective treatment of AMD. Methods: We developed a quantitative surface proteomics approach to survey the apical and basolateral surfaces of RPE primary cultures. We identified targets of interest in angiogenesis via bioinformatics and used CRISPR to test their role in angiogenesis measured as sprouting from choroid explants. We performed intravitreal and subretinal injections to measure choroidal neovascularization induced by matrigel in mice. Results: the polarized surface RPE proteome revealed basolateral receptors and cell-cell communication proteins. We identified receptors and ligands of the plexin and semaphorin families respectively. We confirmed the presence of Semaphorin-4A (Sema4A) in RPE, and Plexin-D1 in choroid (mouse) since this Sema-Plexin pair regulates angiogenesis in other systems. To test whether Sema4A produced by the RPE inhibits choroid angiogenesis, we knocked-out Sema4A in cultured RPE cells and measured their ability to stimulate angiogenesis in co-culture with choroid explants. We found that Sema4A knockout RPE cells had an enhanced capacity to stimulate choroid angiogenesis, indicating a baseline inhibitory role of Sema4A. Finally, we found that exogenous recombinant Sema4A prevented VEGF-induced angiogenesis in choroid explants, and reduced matrigel-induced choroidal neovascularization in vivo. Conclusion: Sema4A is an anti-angiogenic protein secreted by the RPE that inhibits choroidal neovascularization. These results highlight the potential of RPE-choroid cross-talk via Sema-Plexin signaling to counteract the angiogenic effect of VEGF during AMD.

P839/B860

Retinoschisin Protein Therapy for X-linked Retinoschisis Using a Novel Drug Delivery System.

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Background and Objective: X-linked retinoschisis (XLRS) is an inherited retinal degenerative disease caused by mutations in the RS1 gene that leads to splitting of the neurosensory retina resulting in reduced visual acuity in affected patients. RS1 gene encodes a secretory retinoschisin protein implicated in cell-cell interactions and adhesion between photoreceptors and bipolar cells of the retina and also

reported to be expressed in pineal gland for its unexplored role. The prevalence of the disease is 1 in 10,000 worldwide and leads to poor quality of life. Till now, there is no cure for this disease and prophylactic treatment has not been beneficial which often results in severe long term vision-threatening complications. On the other hand, protein replacement therapy holds a lot of promise for oculogenetic diseases. However, the challenge is to deliver the protein effectively, in a stable formulation with maximum availability at the target site of action. Therefore, the study aimed to test the efficacy of retinoschisin delivery using a novel drug delivery device and method for controlled and continuous delivery of drugs through the respiratory mucosa in the maxillary sinus bypassing the blood-brain barrier in mice towards protein therapeutics for XLRS. **Methodology:** Recombinant retinoschisin protein with N-terminal 6xHis tag and lignocaine as control drug were administrated directly into the connective tissue of the maxillary sinus mucosa using the device through an incision made over the nasal bone in wild type mice. Retinal tissue along with brain, liver and serum of the treated mice were processed and analyzed by reversed-phase high-performance liquid chromatography (HPLC) for assessment of delivery. **Results:** the injected retinoschisin protein and the lignocaine drug were rapidly absorbed from the delivery site through the neural lymphatic and vascular route. Significant levels of the protein and drug were detected in the retina as well as in brain within 15 mins, whereas, the bioavailability in liver and serum was undetectable substantiating no systemic leakage. Further histopathological examination of the tissues proved that the delivery modality did not show any toxicity in the treated mice. **Conclusion:** Taken together, these data suggest that the investigated retinoschisin protein therapy can offer effective protein replacement treatment technique for XLRS as an alternative to gene therapy. With more optimized nanoformulation for retinoschisin protein and targeted delivery, the study may open up new prospects to improve the treatment regimen of retinoschisis. Upon successful outcome, this potentially advantageous protein therapeutic approach could be extrapolated to other retinal dystrophies and also to brain disorders.

P840/B861

Preventive Effect of Dopamine on Hyperglycemic Memory-induced Vascular Leakage in the Retina of Diabetic Mice.

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The occurrence of hyperglycemic memory (HGM) is recognized as a phenomenon related to the persistent effects of diabetic vasculopathies even after adequate handling of glycemia. The notion of 'memory' is define that the hyperglycemic environment can be remembered in the vascular system. However, the cause of the hyperglycemic memory phenomenon is unclear. We investigated potential candidate underlying the pathogenesis of diabetic retinopathy via a HGM phenomenon. Our results indicated that it can be predicted low levels of dopamine by reducing tyrosine hydroxylase (TH), a marker for dopaminergic neurons, in the retinas both of diabetic and HGM mice. Although a reduction in dopamine content has been observed in diabetes, its effects in the development of HGM phenomenon-induced retinopathy remains unknown. We found that dopamine prevents HGM phenomenon-induced intracellular events including inflammation, apoptosis and vascular permeability in the human retinal endothelial cells and in the retinas of HGM mice. Our findings suggest that dopamine is a potential drug for treating HGM induced diabetic retinopathy.

P841/B862

Mesenchymal Stem Cells Attenuate Diabetic Kidney Disease through Nadph Oxidases Dependent Mechanism.

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Objective: Diabetic kidney disease (DKD) is one of the major complications of diabetes. Diabetes, among several pathological conditions, alters the redox balance resulting in oxidative stress. The major intracellular sources of reactive oxygen species (ROS), including NADPH oxidases (NOX), specifically NOX 4, previously known as RENOX, cytochrome P450s of the 4A family (CYP4A) and its metabolite 20-HETE, have been shown to be implicated in the pathogenesis of DKD. An emerging body of evidence has revealed that stem cells treatment improves acute tubular injury and induces glomerular repair. However, this mechanism of repair has not yet been identified. This study aims to determine the effect of mesenchymal stem cells (MSCs) treatment on oxidative stress, and to investigate the mechanistic pathway by which MSCs attenuate renal injury.Materials and Methods: Sprague-Dawley rats were divided into two groups: a control group and a streptozotocin-induced type 1 diabetic group each treated with either saline, MSCs-derived medium, or $1x10^6$ MSCs. All injections were administered intravenously. After eight weeks of treatment from diabetes onset, functional, histological and biochemical parameters of the kidneys were assessed. Dihydroethidium (DHE) stain was used to determine ROS production levels. Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Western blotting were performed to determine mRNA levels and protein expression of fibronectin, laminin, Nox4, and TGF-β. The histopathological alterations were assessed using PAS and Masson Trichrome stains.Results: MSCs treatment restored normal urinary albumin excretion levels. Protection against DKD imparted by MSCs was indicated by decreased glomerulosclerosis and tubulointerstitial fibrosis. Moreover, MSCs treatment attenuated tubulointerstitial changes observed in the diabetic milieu. The expression of fibronectin and laminin was significantly decreased in MSCs-treated diabetic rats compared to vehicle-treated diabetic rats. A reduction in oxidative stress was also paralleled by a decrease in the NADPH oxidases activity, NOX4 mRNA and protein expression. TGF- β expression known to mediate cellular hypertrophy and stimulate extracellular matrix biosynthesis in diabetes mellitus was also decreased in the diabetic treated animals.Conclusion: Our results suggest that MSCs have a potential therapeutic effect in the treatment of DKD by attenuating NADPH oxidases-induced ROS production.Keywords: *Diabetic Kidney Disease, Mesenchymal stem cells*, *NOX4,* TGF- β, ROS

P842/B863

Apolipoprotein A-IV Improves Glucose Homeostasis by Acting on Islets and Reprogramming Intestinal Epithelial Cells.

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Roux-en-Y gastric bypass surgery (RYGBP) is one of the most effective procedures for the treatment of severe obesity and type 2 diabetes mellitus (T2DM). The underlying mechanism for the improvement of glucose homeostasis remains unknown. We found that apolipoprotein A-IV (apoA-IV) was significantly increased in patient serum after RYGBP by proteomic analysis and Western blot. The intraperitoneal glucose tolerance test (IPGTT) had been improved dramatically in apoA-IV-treated diabetic animal models (i.e., GK rats, ob/ob mice, and db/db mice). In addition, the hyperglycemia was ameliorated remarkably in apoA-IV-overexpressed ob/ob mice transfected with adeno-associated virus (AAV).

Notably, the serum high-density lipoprotein (HDL) concentration was higher compared with the control group, while low-density lipoprotein (LDL) was lower. To determine possible regulation of apoA-IV expression, we constructed a screening system using CRISPR/Cas9 technology for searching compounds that regulate the apoA-IV expression. We found that glucose upregulated apoA-IV expression in the system. Importantly, we identified that apoA-IV promoted insulin release in two ways under high glucose stimulation condition. One was that apoA-IV directly acted on islets to stimulate insulin secretion, and was also effective on isolated human islets. The other is that IEC6 cells, intestinal epithelial cell, may be reprogramed to β-like cells through upregulating PDX1 expression to release insulin. These results shed light on the functions of apoA-IV in improving glucose homeostasis and provide evidence that apoA-IV may serve as a promising drug for treating diabetes and cardiovascular disease.

P843/B864

Tissue-restricted AMPK Over-expression Mitigates Disrupted Glucose Metabolism in a *Drosophila* Model of Cardiometabolic Disease.

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A worsening global health crisis, cardiometabolic disease involves disrupted lipid and glucose metabolism across adipose, muscle, and cardiac tissue. AMP Kinase (AMPK) is a major regulator of key CMD pathways, such as oxidative stress, fatty acid oxidation, and glucose metabolism. It regulates energy metabolism in adipose tissue, the major lipid repository, and in skeletal muscle, where 80% of glucose uptake occurs. While the protective role of AMPK in CMD has been suggested, AMPK's molecular mechanisms in specific tissues affected by CMD remain poorly understood. We investigate the ameliorative effects of tissue specific AMPK overexpression in a diet-induced *Drosophila* model of CMD. Tissue specific expression of AMPK in striated muscle and in adipocytes is acheived by crossing GAL4 driver lines to a UAS-AMPK line, and to a w^{118} background line as a control. To assess cardiac function and structure, we perform semi-intact cardiac tube preparations followed by analysis of beating heart videos to quantify functional parameters and immuno-staining. We also assess hemolymph glucose levels via enzymatic assay, thoracic muscle structure via bisection and immuno-staining, and lipid accumulation via Nile Red staining. Cardiometabolic dysfunction-higher heart rates and arrhythmicity, lower diastolic/systolic ratio, higher hemolymph glucose levels, and abnormal cardiac structure-was induced with a high-fat (HF) or high-sugar (HS) diet, and verified against controls on a normal diet. AMPK over-expression in muscle decreased glucose by 82%; over-expression in adipocytes decreased glucose by 65%, relative to controls on the same HF and HS diet ($p<0.05$). AMPK in muscle mitigated skeletal muscle atrophy and disarray, contributing to improved flight ability. It ameliorated cardiac dysfunction: diastolic/systolic ratio increased 111%, arrhythmicity decreased 67%, and heartrate decreased 37% ($p<0.05$). Qualitatively, both AMPK in muscle and in adipocytes decreased lipid accumulation in adipocytes surrounding the heart and preserved myocardial structure. These findings implicate AMPK in ameliorating diet-induced disruptions to lipid and glucose metabolism, cardiac function, and cardiac structure: likely mediated through its role as a master regulator in metabolic pathways, warranting further investigation into the AMPK pathway in CMD as a promising therapeutic avenue.

P844/B865

Evaluation of the Impact of *Shpk* Elimination on Macrophage-mediated Hematopoietic Stem and Progenitor Cell Transplantation Therapy for Cystinosis.

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Cystinosis is an early onset lysosomal storage disorder caused by mutations of the *CTNS* gene and characterized by the defective export and subsequent accumulation and crystallization of cystine within lysosomes. Since *CTNS* is ubiquitously expressed, cystinosis presents as a multi-systemic disorder with symptoms ranging from renal dysregulation to abnormalities in the eye, muscle, and thyroid, ultimately leading to premature death. Previous studies found that hematopoietic stem and progenitor cell (HSPC) transplantation into *Ctns*$^{-/-}$ mice prevents disease progression via HSPC-derived macrophage-mediated delivery of functional lysosomes to diseased cells. While Phase I/II clinical trials for this therapy are underway, one potential complication is that roughly 40% of patients with cystinosis carry the 57-kb homozygous deletion eliminating *SHPK* in addition to *CTNS*. *SHPK* is a Pentose Phosphate Pathway (PPP) metabolic enzyme found to directly modulate macrophage polarization. Taking into account that the mechanism of our stem cell-based therapy relies upon macrophages, understanding if it requires *SHPK* holds clinical relevance as it helps elucidate if patients carrying the 57-kb deletion at the homozygous state are likely to benefit from the therapy. To address this question, we studied the impact of *Shpk*$^{-/-}$ HSPC donors on disease rescue following transplantation into *Ctns*$^{-/-}$ recipient mice. We generated and characterized the first *Shpk*$^{-/-}$ mouse model at the genomic, RNA, and protein levels. Mass spectrometry revealed a metabolic phenotype observed as dysregulation of PPP metabolites in *Shpk*$^{-/-}$ livers, while RNA quantitation also showed changes in macrophage polarization markers by cultured primary bone marrow-derived macrophages (BMDMs). We then transplanted *Shpk*$^{-/-}$ HSPCs into lethally-irradiated *Ctns*$^{-/-}$ mice and evaluated transplantation efficacy. We found that *Shpk*$^{-/-}$ HSPCs were effective in the restoration of *Ctns* expression and reduction of cystine levels across multiple *Ctns*$^{-/-}$ tissues as compared to negative *Ctns*$^{-/-}$ HSPC controls. Renal function was also improved in the *Shpk*$^{-/-}$ HSPC-transplanted *Ctns*$^{-/-}$ mice as opposed to the *Ctns*$^{-/-}$ HSPC-transplanted mice. In conclusion, our studies demonstrate that *Shpk*$^{-/-}$ HSPCs may benefit all patients regardless of *SHPK* genotype, confirming that our novel HSPC therapy remains effective at preventing cystinosis.

P845/B866

Upgrading and Characterization of Heart Homing Peptides for Therapeutic Delivery in Cardiomyocytes.

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Cardiovascular diseases represent the major cause of death in western industrialized countries. In particular, 50% of heart failure patients die within 5-years. Although usual treatments exist (*e.g.* β-blockers, mineralocorticoid receptor antagonists), they impact the quality of life due to side effects and off-targets. Thus, targeting specifically cardiomyocytes for drug delivery would certainly represent an improvement over classical ways of administration. Interestingly, two peptides were previously described to exhibit a preferential cardiac tropism (*i.e.* CTP: APWHLSSQYSRT and PCM:

WLSEAGPVVTVRALRGTGSW). However, CTP and PCM peptides efficacy in terms of cell penetration remains low and to our knowledge no optimization has been reported. The aim of this work was to optimize original CTP and PCM peptides and to characterize their respective uptake efficacy and cytotoxicity in cardiomyocytes. Peptide design strategies were carried out (*e.g.* substitution and retro-inverso analysis, cyclization and conjugation to macromolecules or polymers sequences). Over 80 different CTP- and PCM-derived peptides coupled with 6-carboxyfluoresceine (6-CF) were developed. All constructs were assessed for cellular uptake in neonatal rat ventricular cardiomyocytes (NRVC). Cellular uptake efficacy and corresponding viability were quantified by flow cytometry as percentage of 6-CF-positive cells. Staining with fluorescence-based Invitrogen LIVE/DEAD probe was used to evaluate cytotoxicity. Two of the derived peptides, 6-CF-CTP1 and 6-CF-PCM2, exhibited, respectively, a 6.5- and 5.5-fold increase over original 6-CF-CTP and 6-CF-PCM sequences in live cellular uptake. None of the original or derived 6-CF-CTP and 6-CF-PCM-peptides exhibited cytotoxicity when tested at concentrations ranging from 1 to 50 µM. We also performed 4°C incubations which did not prevent the NRVC uptake, excluding an energy-driven process. By high content imaging experiments, we show that these 6-CF- original and derived peptides localized in the cytoplasm. In addition, we are going to identify their location by counterstaining cellular compartments. An alysis will be achieved by confocal microscopy. This study displays potential for optimization of the CTP or PCM peptide sequences to increase their cellular uptake efficacy without cytotoxicity. It raises the potential use of heart targeting peptides for cardiac drug delivery.

P846/B867

Silibinin Administration Ameliorated Pulmonary Arterial Hypertension in a Rat Disease Model.

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Background: the pathogenesis of pulmonary arterial hypertension (PAH) involves endothelial dysfunction, chronic inflammation, smooth muscle proliferation, and pulmonary arteriolar occlusion, and no effective treatment to treat its pathogenesis has been discovered. We previously reported that C-X-C chemokine receptor type 4 (CXCR4) expression in the pulmonary artery (PA) was enhanced (Experimental and Therapeutic Medicine 2018). Therefore, we hypothesized that silibinin, a CXCR4 inhibitor, may decrease PA pressure in the rat PAH model. **Purpose:** We investigated the following hypotheses: 1) CXCR4+ cells migrate to the injured tissue from the bone marrow through the stromal derived factor (SDF-1)/CXCR4 axis, in which SDF-1 is a ligand of CXCR4, and CXCR4+ cells may contribute to the development of PAH. Thus, silibinin administration may improve the pathogenesis of PAH. 2) If CXCR4 is involved in its pathogenesis, silibinin may affect the cells in the bone marrow, as CXCR4+ cells rest in the bone marrow with binding to the niche cells, which are known to express SDF-1. **Methods:** We established a rat PAH model by injecting monocrotaline and maintaining a hypoxic (10% oxygen) environment. Silibinin was suspended in 0.5% carboxymethyl cellulose (CMC). Silibinin or CMC was given orally every day for 5 weeks. We examined the gene and protein expression levels of CXCR4 and other stem cell markers in the PAs by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry each week. We further examined CXCR4 expression in cultured bone marrow cells using RT-qPCR and flow cytometry after 2 weeks of treatment. **Results:** During PAH development, especially in the early stage, silibinin had a treatment effect on PAH development. The CXCR4 gene expression in the PA was also suppressed by the silibinin treatment. However, the gene expression level of CXCR4 was upregulated in the cultured bone marrow cells. The cell population of the

CXCR4+ cells in the cultured bone marrow cells was also increased by the silibinin treatment. Such changes were not observed in the PAH model rats without silibinin treatment. **Conclusion**: Taken together, silibinin can be used as a medicine for PAH. The decrease of CXCR4+ cells in the PA is considered to lead to PAH amelioration. The present study suggests that silibinin affects not only the pulmonary tissue but also the cells in the bone marrow. How and to what extent the effect of silibinin in the bone marrow affects PAH remains unclear.

P847/B868

Therapeutic Potential of Metformin in Novel COPD Mouse Model with Diffuse-type Emphysema.

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Chronic Obstructive Pulmonary Disease (COPD) is a refractory respiratory disease mainly characterized by inflammation, cellular senescence, emphysema and lung dysfunction. Although COPD will be the 3rd leading cause of death in the world by 2030, there is no effective treatment for it. Previously, we have established a novel COPD mouse model, that overexpresses airway-specific epithelial Na^+ channel β subunit (βENaC-Tg) (*Sci Rep.* 2016). However, the usability of βENaC-Tg mice compared with existing COPD mouse models (*e.g.* elastase-induced COPD model) is unexplored. First, to evaluate the histological differences, we developed whole lung image-based quantification for histological analysis. We found that βENaC-Tg showed homogeneous emphysema in the whole lung. Next, we estimated the biochemical differences in both models. Interestingly, we found that βENaC-Tg mice, but elastase-induced COPD mouse model, exhibited changed mRNA expression of inflammatory and senescence-related markers at emphysema-manifestation stage. We demonstrated that βENaC-Tg mice exhibit diffuse-type emphysema with stable expression of inflammatory and senescence-like markers compared to elastase model (*J Phamacol Sci.* 2019). We next verified the therapeutic potential of metformin. In our previous study, we showed that high fat diet-induced type 2 diabetes exacerbates the COPD phenotype and advocated the concept of "pulmonary insulin resistance". Therefore, metformin, a clinically available anti-diabetic drug that is known to improve insulin resistance, could have therapeutic potential for COPD. Administration of metformin (free-drinking water) ameliorated pulmonary emphysema and respiratory function (Compliance, Elastance, forced expiratory volume % in 0.1 second) in βENaC-Tg mice. Next, we examined the effect of metformin on ENaC activity using β/γENaC-overexpressing airway epithelial cells. We showed that metformin suppressed ENaC activity. Therefore, our data imply that metformin could be a novel drug for COPD through the suppression of ENaC. Taken together, these data show the usability of βENaC-Tg mice as a diffuse-type emphysema model and confirm the therapeutic potential of metformin for COPD.

P848/B869

Contribution of the Inflammatory Response during Trauma to Endothelial Cell Permeability.

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Background: Blood loss during trauma resulting in hemorrhagic shock (HS), induces a myriad of physiological responses in the vasculature, leading to endothelial barrier disruption. Endothelial rupture

releases components that magnify the inflammatory response initiated by trauma. If unregulated, the loss of physiological homeostasis can become increasingly deleterious, inducing multiple organ failure and dysfunction, and/or death. Herein, we utilized endothelial cell adhesion and permeability as markers to assess a model for therapeutic treatments that can alleviate or counteract endothelial damage in inflammatory conditions. **Methods:** Human endothelial cells were seeded at two different densities on an electrode-covered E-Plate96 for cell impedance assays. Following 24 hr. Of growth and 1 hr. serum starvation, cells were treated with either media (control), or with varying concentrations (10-30%) of different treatments including lactated ringers (LR), banked porcine baseline serum (BSLN), or banked porcine trauma serum (TM). Continuous cell impedance was recorded for 24 hr. with the xCELLigence® RTCA SP System. Additionally, endothelial cells seeded on Transwell® inserts were used to determine cell permeability following similar treatments; barrier integrity was measured by FITC-Dextran 40KDa. **Results:** Cell index from endothelial cells treated with 10% and 30% TM serum sharply decreased, while the cell index of those treated with the same percentages of BSLN serum remained stable for a longer period of time. Moreover, the sharp cell index decrease observed in cells treated with TM serum occurred much quicker (50hr) compared to BSLN serum (57hr) or LR treatment. Cell permeability indicated that control cells retained barrier integrity, while cells exposed to LR or TM serum leaked dye to the lower chamber, suggesting disruption of the cellular membrane. **Conclusions:** Data from cell impedance and permeability suggest that the components present in serum following trauma disrupt endothelial cell attachment by destruction of cell adhesion properties or cell death. We will continue developing assays to better model HS in *vitro,* evaluate its effect on cellular populations and evaluate potential therapeutics which target inflammatory components to determine their protection of the endothelial barrier. Candidate therapeutics will be further tested in animal models.

P849/B870

Protease Activated Receptor 2 (PAR_2) Expressed on Myeloid Cells Mediates Histaminergic Itch in Male Mice.

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It is known that Protease-activated receptor type 2 (PAR_2) mediates pain and itch through specific action on sensory neurons; here we have shown that myeloid cells expressing PAR_2 are involved in itch responses. Using a novel PAR_2 floxed genetic model, we created a cell specific knockdown of PAR_2 in Lysozyme M $(LysM)^+$ cells, crossing LysMcre and PAR_2 floxed animals ($LysMcre^{+/-}$:$PAR_2^{fl/fl}$). $LysMcre^+$ cells that express PAR_2 are neutrophils, macrophages/monocytes, and mast cells. Flow cytometric analysis from isolated peritoneal cavity and bone marrow immune cells from transgenic animals showed that $LysMcre^{+/-}$:$PAR_2^{fl/fl}$ macrophages and mast cells had significantly reduced expression of PAR_2 in male and female animals, validating our genetic model. We then tested the hypothesis that PAR_2 specifically on $LysM^+$ cells mediated pain and itch in mice. After an intraplantar injection of the PAR_2 agonist, 2-aminothiazol-4-yl-LIGRL-NH_2 (2-at), we observed that mechanical hypersensitivity was no different in subjects lacking PAR_2 on myeloid cells when compared with control littermates, leading us to conclude that direct activation of PAR_2 via 2-at on myeloid cells is not important for mechanical hypersensitivity. We also confirmed that direct PAR_2 activation itself does not cause itch: animals that received an intradermal injection of 2-at did not exhibit an itch response. However, PAR_2 plays a sex-specific role in mediating histaminergic itch in males only. Specifically, itch after mast cell degranulation precipitated by an intradermal dose of compound 48/80 administration. Male mice with PAR_2 knocked down in LysM

cells exhibited a significantly reduced number of scratch bouts. Our findings suggest a novel interaction between mast cell degranulation and PAR_2 on myeloid cells in male animals. Together, these studies represent an important loss-of-function approach to further improve our mechanistic understanding of PAR_2 modulation of pain and itch in the nervous system. This work begins to reveal new mechanistic insight that will have an impact on the design of therapeutics targeting this system.

P850/B871

***In Situ* Immunodetection of Activated Par-2 in Human Skin Reflects Barrier-disrupting Effects of Scratching and Exogenous Protease Activity.**

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Protease-activated receptor-2 (PAR-2) plays an important role in epidermal inflammation and permeability barrier homeostasis. Activation of PAR-2 by proteolytic cleavage at the N-terminus leads to intracellular calcium increase and inflammatory cytokine production. In the granular layers of the epidermis, the calcium increase inhibits lamellar body secretion and delays recovery of the skin's permeability barrier function. In atopic dermatitis, the itch-scratch cycle is the main reason for the exacerbation of the disease. Exogenous proteases, such as dust mite allergens, can easily penetrate the skin after scratching, or if the barrier function is otherwise impaired. On the other hand, the stratum corneum and epidermis contain serine proteases such as KLK14, which is increased in inflamed epidermis. The aim of this work was to examine the roles of endogenous and exogenous proteases in barrier disruption by evaluating the PAR-2-activating effects of scratching and of topical protease (trypsin) application on human skin. For this purpose, we employed immunohistochemistry using an antibody that specifically recognizes N-terminally cleaved (activated) PAR-2. In all cases, positive staining was detected only in the uppermost layer of the epidermis, in the first layer of the stratum granulosum, suggesting prolonged activation of PAR-2 by proteases in the stratum corneum or from external sources. Using ex vivo human skin, we compared the levels of activated PAR-2 in skin sections before and after scratching or topical application of trypsin. Increased immunoreactivity of the N-terminally cleaved form of PAR-2 was observed, confirming that both treatments activate PAR-2. This method might be useful to evaluate the inflammatory status of the skin in patients with atopic dermatitis or similar skin disorders.

P851/B872

Collaborative Development of Therapeutics for Sialic Acid Storage Disease.

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Lysosomal sialic acid (SA) storage disease (SASD) is an autosomal recessive, neurodegenerative, multisystemic disorder caused by defects in the lysosomal SA membrane carrier *SLC17A5* (Sialin). SLC17A5 defects cause free SA and secondary metabolites to accumulate in lysosomes. The clinical spectrum ranges from severe infantile onset in infantile sialic acid storage disease (ISSD; elevated urine SA; ~40 reported cases), to a mild, adult form, called Salla disease (moderate elevation of urine SA; ~120 cases). Although sialic acid metabolism, membrane transport, and lysosomal biology have been extensively studied, the pathobiology of SASD remains poorly understood. Moreover, SASD is likely

underdiagnosed; known patients have experienced a diagnostic delay due to the rarity of the disorder, non-specific clinical symptoms and absence of routine urine SA testing. There is no approved therapy for SASD. As is typical for orphan diseases, the small population of patients makes it difficult to motivate industries to invest in performing the pre-clinical and clinical studies necessary to develop therapies. On the other hand, multidisciplinary collaborative efforts involving the NIH, academic clinical scientists, and patient advocacy groups have successfully overcome the scientific, clinical and financial challenges facing the development of new drug treatments for rare diseases. Encouraged by these successes, we have initiated a collaborative effort for SASD. This has allowed us to start creating cell and mouse models, perform basic/translational research, initiate a natural history study to aid in the identification of biomarkers and treatment endpoints, raise awareness for SASD, and investigate leads on drug candidates. We aim to collect data that incentivize industry to further develop, obtain approval, and commercialize SASD treatments.

P852/B873

Biophysical/biochemical Characterization of the Human Chitooligosaccharide Deacetylase.

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The human chitooligosaccharide deacetylase (HCD), belongs to the carbohydrate esterases family 4 (CE4), is found in various organisms from bacteria to human. Putatively, the HCD catalyzes the deacetylation of acetylated carbohydrates and has been considered to play an important role in the degradation of oligosaccharides. It has been known that the HCD is involved in several inflammatory diseases including ulcerative colitis, psoriasis, and Crohn's disease. Recently, it was also revealed that the HCD is involved in pathological progress of lung cancer such as invasion and migration. Therefore, inhibition of this enzyme can be a novel strategy to prevent the progression of inflammatory diseases and lung cancer. Here, we report the biophysical and biochemical characterization of the HCD. Our analytical ultracentrifugation (AUC) results showed that HCD is a monomer in solution. Among the various acetylated carbohydrates, N-acetyl-D-galactosamine was found to be the most potent substrate candidate using fluorescence-based enzyme activity assay. Next, based on the induced coupled plasma mass spectroscopy (ICP-MS) results and enzyme assay, manganese ion is most active metal cofactor of HCD. We also found that the Asp13 of HCD was directly involved in deacetylase activity. Our results provide insight into its biological characteristics in cellular signaling pathway as well as pathological progress such as inflammatory diseases and cancer. We also performed HTS inhibitor screening of the enzyme using 1,922 compounds of FDA-approved library and 4 hits were selected as the deacetylase inhibitors. This research might open a breakthrough to application of this protein as a potential biomolecule for drug target in cancer treatment.

P853/B874

An Omics-based Study of the Role of C3dg in Keratinocytes: Rnasequencing, Antibody-chip Array, and Bioinformatics Approaches.

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Previously, we have identified the C3dg protein as an important player in the pathogenesis of atopic dermatitis (AD). In this study, we aimed to identify critical factors associated with C3dg in human keratinocytes based onhigh-throughput screening (HTS) approaches.We overexpressed C3dg in HaCaT

human keratinocytes and conducted serial HTS studies, including RNA sequencing analysis integrated with antibody-chip arrays and implementationof bioinformatics algorithms (PPI mappings). Cumulatively, these approaches identified several novel C3dg-associated genes and proteins that are thought to be significantly involved in skin diseases including AD. These novel genes and proteins included LPA, PROZ, BLK, CLDN11, and FGF22, which are believed to play important roles in C3dg-associated skin functions in keratinocytes, as well as genes related to the two importantpathways of systemic lupus erythematosus and Staphylococcus aureus infection. In particular, FGF22 is a unique gene that was detected and validated in all methods applied in this study. By integrating the datasets obtained from these HTS studies and utilizing the strengths of each method, we obtained new insights into the functional role of C3dg in keratinocytes. The approach used here contributes to clinical understanding of C3dg-associated applications and may also be applicable to treatment of AD.

P854/B875

***Daf-16* Plays a Critical Role in Metformin Mediated Anti-aging Effects in *C. Elegans*.**

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The biguanide compound metformin is the first-line treatment for people with type 2 diabetes and one of the most widely prescribed drugs in the world. Besides its anti-diabetic properties, metformin has pleiotropic effects in a variety of cellular processes and pathological conditions. Most prominently, a growing body of evidence in model organisms supports the role of metformin in delaying aging and improving health span, and several large randomized clinical trials are currently ongoing to evaluate the protective effects of metformin in aging and age-related diseases. Despite extensive research in recent years, the exact mechanisms underlying the anti-aging effects of metformin remain elusive. To further elucidate the molecular pathways on which metformin acts to achieve its pro-longevity effects, a genetic screen was performed in the model organism *C. elegans* using strains carrying loss-of-function mutations in key genes of several conserved longevity pathways. Supplementation of metformin hydrochloride (100 mM) increased the mean lifespan for *akt-1(-/-)* from 15.33 to 20.12 days (p<1.0e-6), for *jnk-1(-/-)* from 15.51 to 18.77 days (p=4.4e-5), and for *hsf-1(-/-)* from 10.50 to 11.79 days (p=7.3e-6), indicating that these genetic pathways are not required for metformin mediated lifespan extension. By contrast, no significant difference in mean lifespan was observed for *daf-16(-/-)*, indicating that the anti-aging effects of metformin are in part modulated by *daf-16*. Surprisingly, a profound decrease in lifespan induced by metformin supplementation was observed for *age-1(-/-)* (from 38.54 to 22.74 days, p<1.0e-6) and *daf-2(-/-)* (from 40.63 to 21.54 days, p<1.0e-6), suggesting that metformin may bypass the function of *daf-2* and *age-1* to modulate the activity of *daf-16*. The metformin mediated lifespan reduction in *age-1(-/-)* and *daf-2(-/-)* was reproduced in an independent study and found to be dose-dependent (0-100 mM). Taken together, our genetic analysis not only reveals that metformin prolongs *C. elegans* lifespan in a *daf-16*-dependent manner, but also suggests a novel signaling pathway modulating *daf-16*. Further investigations involving transcriptome analysis are currently underway to understand the underlying mechanisms.

Adipocytes and Metabolism

P855/B876

Study of Anti-obesity Effect of Purple Corn Cobs Extracts in 3t3-l1 Preadipocytes Cells.

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Purple corn cobs is known for its anti-inflammatory and anti-microbial effects. In this study, we determined the effects of ethanol extract of Purple corn cobs on anti-obesity activities in 3T3-L1 preadipocytes. In *vitro* experiments to prevent obesity and improve anti-obesity effects, research using 3T3-L1 preadipocytes is essential, it also distinctly represents morphologic and molecular changes. Our results showed that Purple corn cobs extract decreased the differentiation of 3T3-L1 preadipocytesusing oil red O staining through decreasing the lipid droplet and had no cytotoxicity in 3T3-L1 preadipocytes using WST-1 assay (100 μg/ml and 200 μg/ml). Also, the concentrations of triglycerides(TG) were gradually reduced in 3T3-L1 preadipocytes cells with Purple corn cobs extract (N: 100%, 100 μg/ml: 64.5%, 200 μg/ml: 38.1%) using ELISA assay. An d Purple corn cobs extract suppressed the Expression of PPARγ, C/EBPα and Acetyl-CoA Carboxylase (ACC) in a dose-dependent manner by Western blot analysis. Taken together, our results demonstrate that Purple corn cobs extract indicates anti-obesity effects in *vitro* situation and has potential uses in chemotherapeutic drugs.

P856/B877

Profiling of MiRna Expression in Exosomes Secreted from Differentiating Adipocytes.

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Exosomes, tiny vesicles with 30-100 nm diameters, are released from a variety of cells to communicate with recipient cells. Exosomes contain microRNAs (miRNAs) which suppress translational level(s) of target gene(s). Our previous proteomic study demonstrated that cultured adipocytes secrete not only classical growth factors but also exosomes during adipocyte differentiation (*FEBS Open Biol*. 2016 6: 816-26). However, it remains unclear what type of miRNA is contained in adipocyte secreted exosomes. To address this, we overviewed the differentially expressed miRNAs in adipocyte secreted exosomes during adipocyte differentiation. First, exosome fractions were isolated from cultured media of 3T3-L1 cells following adipocyte differentiation. We found that differentiating adipocytes secreted distinct amounts of exosomes during adipocyte differentiation. The largest amount of exosome was released just before adipocyte differentiation (DM0). The second peak was observed at day 4 following differentiation (DM4). Next, to investigate the expression levels of exosome-derived miRNA, microarray analysis was conducted. The numbers of detected miRNA were 329, 426 and 406 at DM0, DM4, and DM12, respectively. Approximately 10 % of identified miRNAs showed more than 2-fold increment (DM12/DM0) with significant differences. Well-known adipocyte-associated miRNAs such as miR-107-3p and miR-103-3p were contained in this category. By contrast, approximately 8% of miRNAs were altered with less than 0.5-fold of DM12/DM0. Expression levels of representative miRNAs were verified by quantitative PCR. Furthermore, gene ontology (GO) analysis using >2-fold-changed exosome-derived miRNAs predicted target genes which were involved in regulation of transcription, cell proliferation, etc. Collectively, our data showed that differentiating 3T3-L1 cells released exosomes which contained different types and amounts of miRNAs.

P857/B878

Analysis of Fgf Signaling in Sebocytes and Adipocytes.

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[Objectives] Sebaceous gland cells (sebocytes) and adipocytes synthesize and store a large amount of lipid in vivo that serve for the functional maintenance of the skin surface and for energy storage/mechanical cushion, respectively. Recently, fibroblast growth factor (FGF) receptor (FGFR) signaling has been reported to be involved in the regulation of lipid synthesis in these cells. Acne formation has been attributed to enhanced FGFR2 downstream signals. FGF2 and FGF10 were reported to regulate adipocyte differentiation and proliferation. In this study, we aimed at clarifying the involvement of FGF signaling in the regulation of lipid synthesis, proliferation and differentiation of sebocytes and adipocytes. [Methods] an immortal SZ95 human sebocytes established from facial sebaceous glands and 3T3-L1 mouse preadipocytes were used. Differentiation was induced by LA in SZ95 cells, or by insulin, IBMX, and dexamethasone in 3T3-L1 cells. The lipid droplets synthesized and accumulated by these cells were stained with either Oil Red O or with Nile Red, and the lipid staining pattern was analyzed microscopically or by flow cytometry. Expression levels of lipid-synthesis related enzymes, FGF ligands, and growth- and differentiation-related genes were determined in the cells treated with fatty acids, FGF ligands, FGFR inhibitors or other compounds. [Results] in SZ95 cells, treatment with linoleic acid (LA) remarkably enhanced lipid synthesis. This lipid synthesis was upregulated by FGF18, and was suppressed by FGF7. In differentiation-inducing culture of 3T3-L1 cells, FGFC (a universal ligand for all FGFR subtypes) or FGF18 abrogated cellular differentiation. Proliferation of both SZ95 cells and undifferentiated 3T3-L1 cells was enhanced by FGF7 and FGF10, but was suppressed by FGF18. LA treatment of SZ95 cells resulted in increased FGF18 expression and decreased FGF7 expression. [Conclusions and Discussion] While proliferation of sebocytes and preadipocytes are similarly enhanced by FGFR2b ligands FGF7 and FGF10, lipid biosynthesis/ adipocyte differentiation is differentially regulated by FGFs with different FGFR specificities. The increased FGF18 gene expression and decreased FGF7 gene expression in LA-treated SZ95 cells suggest that proliferation, differentiation and lipid biosynthesis are inseparably regulated involving FGF signaling pathways.

P858/B879

STIM2β Regulates White Adipose Tissue (WAT) Development.

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Adipose tissue has a specialized role in energy storage and thermogenesis. However, when there is excess lipids over the storage capacity of adipose tissue, excess lipids “spill over” may occur from adipose to non-adipose tissues. Abnormal lipid accumulation in non-adipose tissues such as skeletal muscle and liver, not in WAT, is known to cause lipotoxicity : endoplasmic reticulum stress, increased inflammation response, mitochondrial dysfunction, insulin resistance (IR) and other effects. Therefore, profer regulation of adipogenesis is important for the homeostasis of lipid metabolism. There was several studies that relates SOCE and adipogenesis. However, the detailed mechanism of SOCE on the regulation of adipogenesis remains elusive. In this study, we show STIM2β expression level is increased during adipogenesis and functions as a regulator of adipogenesis. STIM2β KO 3T3-L1 cell line showed increased lipid accumulation during adipogenesis in comparison with WT cell line. STIM2β KO affects to Ca^{2+} signaling on 3T3-L1 cell and leads change in cell cycle. STIM2β KO mouse showed difference in

adipose tissue development in comparison with WT. Our data provide an insight on the importance of intracellular Ca^{2+} concentration regulation by STIM2β on adipose tissue development.

P859/B880

Adipocyte-macrophage Crosstalk Upon the Expression of Catecholamine Synthesis Pathway Genes.

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The crosstalk between adipocytes and macrophages contributes to the systemic metaflammation observed in obesity and associated immunometabolic disease clusters. While adipocytes and macrophages produce, secrete and respond to catecholamines (CA), the underlying mechanisms regulating their synthesis in the interplay between immune and metabolic systems remain unknown. Tyrosine hydroxylase (TH) and phenylethanolamine N methyltransferase (PNMT) are two of the main enzymes responsible for CA synthesis. We hypothesize that under an obesogenic state CA play a role in the crosstalk between metabolic and immune systems. A model of indirect cell coculture with conditioned medium (CM) from RAW 264.7 macrophages (with or without LPS-activation) and 3T3-L1 adipocytes and preadipocytes was created to study the effect of cellular secretomes on CA synthesis pathway. The relative expression (mRNA levels) of TH and PMNT involved in CA synthesis was determined by qRT-PCR. RAW 264.7 macrophages, both LPS-activated and non-LPS-activated, were exposed to CM from preadipocytes and mature adipocytes. These cells were exposed to LPS, interleukin 4 and interferon γ. We also assessed the effect of 3T3-L1 preadipocytes and fully differentiated adipocytes secretomes upon the expression of TH and PNMT in both LPS-activated and nonactivated RAW 264.7 macrophages. We found a decrease of TH and PNMT expression during the adipocyte differentiation process. The secretome from LPS-activated macrophages downregulated TH and PNMT expression in preadipocytes, but not in mature adipocytes. Mature adipocytes CM induced a decrease of PNMT levels in RAW 264.7 macrophages. Pre and mature adipocytes showed a similar pattern of TH, PNMT and peroxisome proliferator-activated receptor gamma expression after exposure to pro and anti-inflammatory cytokines. We evidenced macrophages and adipocytes coregulate the expression of CA synthesis enzymes through their secretome, with a putative role for non-inflammatory signaling pathways. Mediators released by macrophages seem to equally affect CA production by adipocytes, while adipocytes secretome preferentially affect adrenaline production in macrophages. CA synthesis regulation seems to be more determinant in early stages of adipogenic differentiation. The discovery that macrophages and adipocytes co-regulate CA synthesis suggest these amines are key signaling molecules connecting immune and metabolic systems regulating immunometabolic disease clusters.

P860/B881

Changes in Gene Expression of Lipid Metabolism Genes in Collaborative Cross Model of Non-alcoholic Fatty Liver Disease.

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Non-alcoholic fatty liver disease (NAFLD) is the most common form of liver disease in the Western world, affecting approximately 30% of the general Western population with strong onset prevalence with metabolic syndrome, obesity, and diabetes. NAFLD, characterized as the accumulation of fat in the liver in the absence of excessive alcohol drinking and secondary liver pathologies, can worsen and progress to non-alcoholic steatohepatitis (NASH) or severe pathologies such as fibrosis and cirrhosis resulting in irreversible liver damage. Currently, the molecular mechanism of NAFLD onset and progression is unknown, likely due to the great genetic diversity of NAFLD patients, urging the discovery of genetic markers that could be non-invasively analyzed in patients. In this study, to mimic the heterogenous population of individuals affected by NAFLD, we utilized the collaborative cross (CC) mouse population, a state-of-the-art model that mimics the genetic diversity of the human population with the added benefit of reproducible genetic backgrounds. Male and female CC mice were fed a high fat/high sugar (HF/HS) diet for 12 weeks and then analyzed for NAFLD prevalence and progression. Similar to human patients, we observed significant diversity between individual mouse strains body weight gain, liver lipid metabolism disturbances characterized by elevated triglyceride (TG) and total cholesterol content, as well as histopathological indicators of NAFLD-associated liver injury. Furthermore, gene expression analysis of lipid metabolism genes revealed not only significant changes in the population wide expression, but also interindividual strain differences in several marked genes regulating lipid accumulation, including *Cd36*, *Fabp2*, and *Ppar-ɣ*. Furthermore, we found that altered gene expression of lipid accumulation regulation genes correlated with the accumulation of liver TG. In conclusion, our model of NAFLD mimics the genetic heterogeneity observed in the human population of NAFLD patients in terms of onset, and severity/progression of the disease. This indicates a great value of the CC mouse model as a strong tool for the identification of non-invasive biomarkers of NAFLD and key molecular pathways that correlate with disease onset and progression.

P861/B882

Prokineticins in Liver Diseases- New Insights of Iron Trafficking Proteins and Dysregulation of Nitric Oxide Behavior in Patients with Non-alcoholic Fatty Liver Disease.

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Prokineticins in liver diseases: new insight of iron trafficking proteins and dysregulation of nitric oxide behavior in patients with non-alcoholic fatty liver disease NAFLD is increasing worldwide, imitating current epidemics of lifestyle diseases. This is escorted by Prokineticins in conjunction with nitric oxide interlinked pathways either vasodilatory effect of NO gene transcription or iron metabolic abnormalities

leading to NAFLD/NASH. Iron trafficking proteins growth differentiation factor-15 and Prokineticins as mediators of inflammatory molecules may have potential role in NAFLD, thus we explored whether GDF-15, iron trafficking proteins and Prokineticins are involved in severity of NAFLD. Blood and biopsy samples were collected from the Gastroenterology, AIIMS, Delhi. Each was screened for serology and categorized into sub-groups, i.e. NAFLD (n=30), Hepatitis (disease control n=38), diabetes mellitus without metabolic syndrome (DM without Mets) (n=20), Metabolic syndrome without diabetes mellitus (Mets without DM) (n=25), and Healthy subjects (n=25). Adiponectin, leptin, Insulin Resistance, BMI, pro-inflammatory and anti-inflammatory cytokine, iron trafficking proteins, Prokineticins and GDF-15 levels were measured in all groups. GDF15 levels, iron trafficking proteins and Prokineticin expressions were higher in NAFLD, DM with MetS, and MetS without DM than in healthy controls. GDF-15 levels may have hidden relations in respect to body weight, waist hip ratio, BMI and HOMA IR. Prokineticins expressions were higher in NAFLD, HCC patients, and DM with Metabolic syndrome than diseases controls (HBV & HCV) and healthy controls. Expressions of Prokineticin-1 have noticed 3-4 fold change in patients with cirrhosis and liver cancer (HCC). Furthermore, increasing pattern of viral load of HBV and HCV infections ($>10^4$ IU/ml) have suggested that the expression of Prokineticins were 3-4 fold in patients with cirrhosis and liver cancer (HCC). Our finding suggests that high circulating levels of GDF-15, iNOS and overexpression of Prokineticins could be potential biomarker of NAFLD, and suppressed levels of antioxidants gene could be linked in development of future diabetes, CVD and metabolic syndrome. Thus, Prokineticins and dysregulation of iron trafficking proteins and NOS expressions could be novel emerging biomarkers to treat NAFLD.

P862/B883

Rapid Induction of Skeletal Muscle Thermogenesis in the Absence of Interscapular Brown Adipose Tissue.

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Obesity is a disorder caused by positive energy balance, where energy intake is greater than energy output, leading to weight gain. One strategy to counter energy imbalance is to increase output, or energy expenditure (EE). Skeletal muscle makes up 40% of the body composition of non-obese adults and is a large contributor to BMR, which accounts for 50-70% of total daily EE. Thus, skeletal muscle is a promising potential therapeutic target for increasing EE passively to promote weight loss. However, this potential of skeletal muscle has been historically less studied as a component to obesity therapy than adipose, especially brown adipose tissue (BAT). We have discovered a neuronal pathway that contributes to a physiological increase in energy expenditure by non-shivering thermogenesis in skeletal muscle when rats are exposed to a predator odor (PO) from ferrets. We predict that PO-induced skeletal muscle thermogenesis can occur independently of BAT. Male (n=8) and female (n=8) Sprague-Dawley rats underwent surgical excision of interscapular BAT (n=4/sex) or sham surgery (n=4/sex), and transponders were implanted in the left and right gastrocnemius muscles. Single-housed rats were habituated to experimental conditions prior to PO and control exposure. PO and control towels (1.5”x2”) were placed in home cages, and PO-induced thermogenesis was measured for 120 minutes after exposure. Activity thermogenesis following PO exposure was measured using a treadmill walking protocol, and calorimetry was used to measure differences in EE. PO exposure induced skeletal muscle thermogenesis similarly in animals with and without interscapular BAT, and there was no significant difference in activity thermogenesis. Similarly, PO exposure did not significantly affect the PO-associated increase in EE. The excision of interscapular BAT, the largest depot in rats, did not impede PO-induced

skeletal muscle thermogenesis or EE. These results do not support the idea that BAT makes a meaningful contribution to PO-induced thermogenesis or EE and implicate skeletal muscle as a potential target for reducing positive energy balance that contributes to obesity.

P863/B884

Impaired Branched-chain Amino Acids Catabolism in White Adipose Tissue of Non-obese an kyrin-b Mutant Mice Contributes to Systemic Metabolic Dysfunction.

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Increased serum levels of branched-chain amino acids (BCAA) in obese humans are one of the most accurate predictors of both risk for developing insulin resistance (IR) and metabolic outcomes of weight loss interventions. However, whether elevated circulating BCAA levels are causative of obesity and metabolic impairment or a response to it is not clear. Likewise, the mechanisms underlying the relationship between elevated BCAA levels and impaired peripheral insulin response remains to be fully elucidated. Young knock-in mice bearing the human type 2 diabetes (T2D)-linked R1788W variant in the cytoskeletal protein ankyrin-B (AnkB) show normal body weight and composition. In addition, these mice exhibit increased glucose uptake by white adipose tissue (WAT) due to deficits in the internalization of the glucose transporter GLUT4. We previously reported that sustained elevations in glucose disposal in WAT lead to the onset of increased adiposity, lipotoxicity, systemic inflammation, and IR in an kB mutant mice with age or in young animals fed Western diets. Transcriptomic analysis of non-obese R1788W an kB mice identified gene transcriptional changes consistent with lower BCAA catabolism in WAT. Here, we show that an kB deficiency impairs BCAA catabolism in WAT prior to the onset of obesity. Lean R1788W an kB mice show downregulation of BCAA catabolic enzymes in WAT, concomitant with elevations in serum BCAA and related acylcarnitines detected by targeted metabolomics. In contrast, an kB deficiency did not alter BCAA oxidation in skeletal muscle. Results from studies in which control and R1788W an kB mice were fed isocaloric low fat (LFD) and high fat (HFD) diets, or similar diets enriched in BCAA, suggest that increases in circulating BCAA levels cause accumulation of catabolic intermediates in skeletal muscle that trigger mitochondrial dysfunction and impair insulin signaling. Similarly, elevated BCAA altered the secretory capacity of pancreatic beta cells and caused endoplasmic reticulum stress. Our data indicates that elevated circulating BCAA impair glucose tolerance and insulin sensitivity independently of obesity, and that these deleterious effects are potentiated by a HFD.

P864/B885

Voluntary Exercise Training Improves Body Weight of Leptin-Deficient ob/ob Mice by Changes in Hepatic Stearoyl-CoA Desaturase 1 and Deleted-in-Breast-Cancer 1 Protein Levels.

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Deleted-in-Breast-Cancer-1 (Dbc1) ablation causes obesity and stearoyl-CoA desaturase 1 (SCD1) induces the biosynthesis of monounsaturated fatty acids. The purpose of this study was to determine whether voluntary wheel running (VWR) alters SCD-1 and Dbc1 protein levels in the liver of leptin-deficient ob/ob mice. Twenty-five Ob/Ob mice were divided into 2 groups (ob/ob-Sed, ob/ob-Ex). After 10 weeks, VWR significantly reduced body weight without affecting fatty acid synthase and CD36 protein levels. The improvement is associated with changes in hepatic SCD1 and Dbc1. We found that

hepatic SCD-1 protein levels were significantly increased and Dbc1 protein levels were decreased in ob/ob-Sed animals; however, the effect was attenuated by VWR. This is the first study showing that VWR has strong effects on hepatic SCD1 and Dbc1 of ob/ob mice and provide key insight concerning the effect of exercise on obesity.

P865/B886

The New Role of Msc1 in Adipogenesis and Its Relationship to Fas.

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Background and aims: Adipocytes play a role in energy homeostasis and process the largest energy reserve as triglycerol stay in a dynamic state, they start expanding when the energy intake is higher than the expenditure and undergo mobilization. Adipogenesis is a tightly regulated cellular differentiation process, in which the preadipocytes are transformed into differentiated adipocyte cells. Recent evidence indicates that adipose tissue is an active endocrine organ which helps to regulate several body functions, including insulin-mediated processes, lipid and glucose metabolism. In addition, adipose tissue plays a role in coagulation and some aspects of inflammation. Previous studies found that there are loss of body weight and fat storage in Metabolic Syndrome complex (MSC1) knockout mice. Based on this evidence, we hypothesized that MSC1 would affect adipogenesis and lipogenesis. **Materials and methods:** in this study, we used 3T3L1 cells, a mouse adipocyte, using western blotting and qRT-PCR to confirm the expression level of adipogenesis, lipogenesis genes and MSC1 expression. Promoter assay have used to identify genes regulated by MSC1. An d, the animal model used MSC1 transgenic mice, and we compared the metabolic changes with Wild type mice on fed high fat diet. **Results:** in our study, MSC1 is upregulated during adipogenic differentiation at an early stage. Also mRNA levels of MSC1 and Fatty acid synthase (FAS) are increased during the same period of adipogenic differentiation. On the third day of differentiation, MSC1 overexpression increased FAS expression together with the acceleration of PPARγ and FABP4 expression. On the contrary, shRNA-mediated depletion of MSC1 resulted in the reduction of FAS, PPARγ and FABP4 expression. In addition, MSC1 is able to directly bind to FAS promoter in 3T3L1 cells. Histological analysis of the adipose tissue revealed hypertrophy in HFD-fed MSC1 transgenic mice, indicating that MSC1 overexpression affects the adipogenesis and obesity. **Conclusion:** Taken together, MSC1 is an important modulator of adipogenesis and lipogenesis. Furthermore, these results suggest that the decreased MSC1 expression that occurs an important role in preventing lipid accumulation and obesity.

P866/B887

A Role for Alms1 (Alström Syndrome 1) in Leptin Release from Adipocytes.

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Inactivating mutations in the ALMS1 gene in humans cause early onset obesity and metabolic syndrome. Deletion of ALMS1 in rodents induces early onset obesity and insulin resistance on normal chow. The mechanisms by which ALMS1 cause obesity are not clear but likely involve the development of leptin resistance since adult ALMS1 Knock out (KO) rats are hyperphagic and hyperleptinemic. However, it is

not clear whether ALMS1 is involved in leptin release and expression in adipocytes prior to development of obesity. ALMS1 has been associated to ciliary function which may play a role in adipogenesis. We hypothesized that deletion of ALMS1 would increase leptin release from fat pads of young ALMS1 KO rats, which should contribute to hyperleptinemia. To study this, we obtained abdominal fat pads from young ALMS1 KO rats (8 weeks) prior to changes in body weight. We found that leptin release (normalized to fat weight) was 120% higher (from 30±8 to 56± 15 pg/mg tissue/2h) in abdominal fat pads from ALMS1 KO rats. Plasma leptin levels were dramatically higher in young ALMS1 KO rats (ALMS1: 3530±300 vs. WT: 172±16 pg/ml, $p<0.05$) despite no significant differences in body weight or baseline glucose. To study the mechanisms behind higher leptin release we performed RNAseq in freshly isolated abdominal fat pads. 12,860 genes were identified and quantified. In fat pads from ALMS1 KO there were 163 upregulated and 237 significantly downregulated genes. Leptin mRNA was significantly increased (1.68 Log2 fold, $p<0.0001$, n=4) as were several genes involved in lipogenesis (Elovl6, Scd1, Scd2, Mogat2, Acly, Lpl, Dgat2, Fasn). Interestingly, at least 10 ciliary genes were downregulated (Traf3ip1, Pard6g, Cngb1, Ak7, Ttll1, Mlf1, Ccdc162, Dnah6, Ccdc187, Foxj1) suggesting a role for ALMS1 in ciliary function. To examine this, we immunolabeled cilia in abdominal adipocytes with gamma acetylated tubulin antibodies, and examined sections by confocal microscopy. Short cilia (1-2 μm) were observed in both WT and ALMS1 KO adipocytes. We conclude that ALMS1 is likely involved in leptin release from adipocytes. It is not clear whether increased leptin expression and release is secondary to neuroendocrine alterations or alterations in cilia-induced signaling in adipocytes. Our data also point to an important role for ALMS1 in regulating adipocyte function.

P867/B888

Homeodomain-interacting Protein Kinase 2 (hipk2) Regulates Ucp1 Expression in Brown Adipocyte through Modulating Pka Signaling and Ppar Activation.

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Homeodomain-interacting protein kinase 2 (HIPK2) is a serine/threonine kinase that influences multiple cellular functions including apoptosis and differentiation through modulation of transcriptional regulators and signaling molecules. Recently, it is reported that HIPK2 is required for white adipocyte differentiation and development. However, the role of HIPK2 in brown adipocytes has not been reported even through HIPK2 expression is enriched brown adipose tissues (BAT). We observed that HIPK2 expression is significantly induced during the differentiation of mouse brown adipocyte cell line (iBPA) in accordance with uncoupling protein1 (UCP1). To assess the role of HIPK2 in brown adipocyte, we generated *HIPK2* knockout (K/O) cells by knocking-in EGFP reporter gene into HIPK2 genome via CRISPR/Cas9-induced homology-independent DNA repair. HIPK2 K/O cells showed dramatic decrease of UCP1 expression whereas expression levels of ACLY, Adrb3, PPAR-α, PPAR-$_{\gamma}$, FASN, Srebf1, ACACA and MOGAT are slightly decreased or increased compared to control cells. Furthermore, we observed that UCP1 induction in response to CL316243, an agonist of β3 adrenergic receptor, is decreased in HIPK2 knockout iBPA cells (D5) and defects of HIPK2 exhibit decreased level of phosphorylated CREB and p38 which is involved in the regulation of UCP1 gene expression under the treatment of CL316243. In addition, HIPK2 increased PPAR$_{\gamma}$-mediated PPRE-tk-luciferase activity. These results suggest that HIPK2 is closely involved in modulating signaling events regarding UCP1 expression. This work was supported by a National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MSIP) (NRF-2017R1A2B4009674, NRF-2017R1A2B4007462 and NRF-2011-0030086)

P868/B889

Ginsenoside Rd Inhibits Lipid Accumulation by Downregulating Adipogenesis and Lipolysis in 3T3-L1 Preadipocytes.

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Obesity, a state of excess fat accumulation in adipocytes, is a growing public health issue in the developed world. Given that ginsenoside Rd, one of major ginsenosides in ginseng (the root of *Panax ginseng* C.A. Meyer) possesses anti-inflammatory and antioxidant activities, it may have potential anti-adipogenic effect. In this study, we investigated whether Rd inhibits adipogenesis and lipid accumulation in 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were induced to differentiate into adipocytes in the presence or absence of Rd for 8 days. Rd suppressed the differentiation and inhibited lipid accumulation as indicated by lower intracellular lipid and triglyceride contents as well as reduced adipogenic gene expression upon treatment. Rd-treated 3T3-L1 cells released more glycerol, indicating increased lipolysis, which was supported by increased expression of lipolysis-related genes, including adipose triglyceride lipase. In conclusion, Rd markedly decreased intracellular lipid accumulation by downregulating adipogenic gene expression and enhanced lipolysis, implying it may have potential as an anti-obesity agent.

P869/B890

***Hydrangea Serrata* (thunb.) Ser. Extract Prevents Hyperlipidemia and Obesity Via Lipid Metabolism Modulation in High Fat Diet-induced Obese Mice.**

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Recently, there has been a growing propagation of obesity caused by global tendency towards reduction of physical activity and an augmented dietary intake of fats, sugars, and high calories. Obesity is a major risk factor of diabetes, cardiovascular diseases, metabolic diseases, and several forms of cancer, such as breast, colon, and prostate. The aim of this study was to examine the anti-obesity effect of hot water extract of *Hydrangea serrata* (Thunb.) Ser. (WHS). To investigate the pharmacological activities of WHS in *vivo*, we examined several obesity-related parameters in high fat diet (HFD)-induced obese mice. Compared to the HFD group, oral administration of WHS showed significant reduction of body weight gain (24.21%, 24.46%, and 31.01% at 75, 150, and 300 mg/kg). The fat-pad weight and the percentage of fat in tissue measured by dual X-ray analyzer were also notably decreased by WHS administration. The expanded size of adipocyte in HFD group were markedly decreased in WHS-administered group. WHS intake also effectively ameliorate the increased serum levels of total cholesterol, low-density lipoprotein (LDL), leptin, and insulin. Moreover, fat accumulation in the liver were distinctly improved by WHS administration. All these results strongly suggest that dietary supplementation of WHS could be helpful in preventing or treating obesity. This work was supported by "Food Functionality Evaluation program" under the Ministry of Agriculture, Food and Rural Affairs and partly Korea Food Research Institute (2019G0190300-01).

P870/B891

Elucidating the Role of TM6SF2 in Nonalcoholic Fatty Liver Disease.

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Nonalcoholic fatty liver disease (NAFLD) is the most common form of liver disease, affecting an estimated 80-100 million Americans. NAFLD is characterized by accumulation of excess fat stored in the liver, which can lead to nonalcoholic steatohepatitis, followed by cirrhosis. Previous exome-wide association studies found an E167K mutation of TM6SF2 is associated with susceptibility to NAFLD. Emerging data show that TM6SF2 promotes secretion of lipidated lipoproteins, preventing the accumulation of triacylglycerols (TAGs) in hepatocytes. However, the precise mechanism by which this occurs is yet to be determined. Structural predictions of TM6SF2 report 7-10 transmembrane domains, with 2 of 6 TOPCONS models placing the C-terminus of TM6SF2 in the cytoplasm. To map the topology of TM6SF2, we performed fluorescence microscopy of cells expressing N- and C-terminally tagged TM6SF2 under conditions in which luminal epitopes were protected. Our results show that the N-terminus of TM6SF2 is in the cytosol, whereas the C-terminus resides in the lumen. Based on both our data and structural predictions, the E167K mutation of TM6SF2 should reside in the lumen. However, further mapping of the transmembrane domains is necessary. To understand the cellular function of TM6SF2, we performed proteomic profiling of tagged TM6SF2 in human hepatoma cells to identify interaction partners. Our proteomics revealed that ApoB100, a key protein in the secretion of VLDLs, interacts with TM6SF2. Whether this interaction is important for lipidation of VLDLs, and whether the E167K mutation affects this process is yet to be determined. Understanding the structure of TM6SF2 and its relationship with its binding partners will provide a more comprehensive understanding of the causes and potential treatments of NAFLD.

Muscle Structure, Function, and Disease

P871/B892

New Players in the Pathogenesis of a Lysosomal Storage Disease.

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There is growing evidence that the pathogenesis of tissue damage in a large group of lysosomal storage diseases (LSDs) cannot be adequately explained by the enlargement of lysosomes filled with undigested materials. This concept fully applies to Pompe disease, a deadly neuromuscular disorder caused by the absence or deficiency of glycogen-degrading lysosomal enzyme acid alpha-glucosidase (GAA). In both the most severe infantile form of the disease and in its so called milder later-onset form, skeletal muscle is a major affected tissue. By using biochemical and molecular analyses, as well as confocal microscopy of live or fixed muscle fibers from our mouse model of the disease, we have shown that the lysosomal entrapment of glycogen initiates a pathological cascade including altered autophagy, mitochondria abnormalities, and, perhaps not surprisingly, changes in the localization and activity of mechanistic target of rapamycin complex 1 (mTORC1) and AMP-activated protein kinase (AMPK), the two critical nutrient-responsive kinases that belong to a lysosome-based signaling system. We have found massive buildup of autophagic debris, a diminished mTORC1 activity, and excessive accumulation and activation of AMPK at the lysosome in the diseased muscle. Furthermore, we challenged the prevailing view in the field that the lysosomal glycogen storage does not lead to metabolic consequences in muscle cells. We

have shown that the metabolome profile of GAA-deficient muscle is reminiscent of that in cells with limited availability of glucose, and that there is a cross-talk between glycogen breakdown in the cytosol (phosphorylytic glycogenolysis) and hydrolysis of glycogen to glucose in the lysosome. Accumulation of the precursor of glycogen synthesis, a decrease in the metabolites of the glycolytic pathway, cellular energy deficit, and a shift to lipids as the energy source are observed in the diseased muscle. Pompe disease is one of several LSDs being treated with enzyme replacement therapy (ERT). The most consistent effect of ERT has been on cardiac pathology and function in infants who, unlike late-onset patients, invariably suffer from severe cardiomyopathy. Despite this considerable progress, the therapy is still not satisfactory since the effect in skeletal muscle is minimal. We have now tested a new experimental drug, AT-GAA (recombinant human GAA; Amicus Therapeutics), with much improved lysosome-targeting properties in a large pre-clinical study. Remarkably, this new drug reversed or significantly improved all aspects of the disease pathogenesis.

P872/B893

Multi-dimensional Mapping of Cell States during Cardiomyocyte Differentiation Using Live Imaging and RNA FISH.

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The Allen Institute for Cell Science is developing a state space of stem cell structural signatures to study changes in cellular organization of human induced pluripotent stem cells (hiPSCs) as they differentiate into cardiomyocytes. We have used CRISPR/Cas9 to generate a collection of ~35 endogenous fluorescently tagged hiPSC lines (www.allencell.org), each expressing a monoallelic EGFP-tagged protein that localizes to a particular cellular structure or organelle. In addition, we have developed several pertinent methods: 1) scarless GFP-tagging of late expressing cardiomyocyte genes, including ACTN2, ssTNNI1, MYL2, MYL7 and TTN, to study the organization and morphogenesis of the contractile apparatus; 2) a robust protocol for differentiation of hiPSCs into cardiomyocytes and methods for preparing cells for imaging; and 3) image-based assays and segmentation algorithms that enable single-cell analyses of structure localization in differentiated cardiomyocytes. Using the cardiomyocyte-specific protein ACNT2 as a reference, we have also developed methods for quantifying the extent of sarcomeric organization in cells across timepoints, thus placing individual cells along a psuedotime axis based on their level of structural ACTN2 organization. We are also using this approach to analyze sarcomere maturation via other key sarcomeric structural and regulatory proteins. This permits us to use sarcomere organization state as a reference system to analyze the reorganization of cytoplasmic structures. We are now incorporating multiplexed fluorescence in situ hybridization (FISH) into this analysis, allowing us to directly investigate the connection between cell structure organization and gene expression profiles on a single-cell level and also to produce structure-referenced maps of RNA localization. This imaging pipeline is generating an image database of high-resolution, high-replicate image data of the fluorescently tagged structures with a goal of generating an integrated image “state space” of intracellular reorganization during cardiomyocyte differentiation.

P873/B894

Investigating the Contribution of Muscle Contractile Apparatus Genes to Muscle Function in *Caenorhabditis Elegans*.

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Caenorhabditis elegans has been widely used as a model organism to study muscle function with their sarcomeres composed of dense bodies and M-lines, organizing and anchoring thin actin filaments and thick myosin filaments to the muscle cell membrane, respectively. Dense bodies and M-lines play substantial roles in muscle contractile apparatus by transmitting the force generated from muscle contraction to the basement membrane and cuticle permitting animals to engage in their undulatory locomotion. To elucidate the genes responsible for muscle performance, we have developed two novel assays that involve exertion of muscle forces by *C. elegans*. First, is a Pluronic gel-based burrowing assay (Lesanpezeshki *et al.,* 2019) that challenges the animals to move in 3D under a chemical stimulus. Second, is a microfluidic pillar-based platform called NemaFlex (Rahman *et al.,* 2018) that quantifies the maximum exertable force by animals. Using these assays, we scored mutants with defects in contractile apparatus from dense bodies and M-lines to thick and thin filaments, and interestingly, burrowing ability proved to be distinct from NemaFlex strength measurements. The key results thus far are: (i) *atn-1* (α-actinin), *uig-1* (Cdc42 GEF), *zyx-1* (zyxin), *tln-1* (talin) and *pfn-3* (profilin) mutants were not different from wild-type animals in NemaFlex scoring of muscle force, while *dyc-1* (capon), *dim-1, unc-95, unc-82* (serine/threonine kinase)*, unc-89* (obscurin)*, unc-54* (myosin heavy chain)*, lev-11* (tropomyosin) and *unc-60* (ADF/cofilin) genes played significant roles in muscle force exertion. (ii) All the tested mutants were impaired in burrowing compared to wild-type animals, highlighting the importance of the genes in muscle function. Interestingly, mutants in α-actinin, ADF/cofilin, and twitchin were among the most defective mutants for burrowing behavior of *C. elegans*. Thus, our assays can reveal phenotypic differences in animals with mutations affecting muscle function. We intend to investigate the structural defects of the sarcomere in these mutants using immunostaining techniques to understand if they can be related to the type of deficiencies detected in our burrowing and NemaFlex assays.

P874/B895

Using Developmental Biology to Identify Treatments for Disease: Crispr/cas9 Ablation of Individual MicroRNAs from a MicroRNA Family Reveals Their Individual Efficacies for Regulating Cardiac Differentiation.

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Developmental Biology can be used to identify treatments for disease. For example, genes involved in heart development have been identified as mis-expressed in heart failure (HF). We recently showed that many of these developmentally related HF genes are regulated by specific MicroRNAs. These small, non-coding RNAs use a 7-8 base-pair seed sequence to target a corresponding mRNA sequence resulting in rapid down-regulation of translation. MiRNAs can also control and promote protein homeostasis, which, if compromised, can result in developmental defects or initiation of disease. Here, we first identify that during differentiation, individual miRNAs that reside in the miRNA17 family do not share the same

function even though they have the same seed sequence. The advent of CRISPR/CAS9 technology has not only yielded a true observation of individual miRNA function, it has also reconnected advanced molecular biology approaches to classical cell biology approaches such as gene rescue. We show that miRNA106a and to a lesser extent miR17 and 93 target, in a dose-dependent manner, the cardiac suppressor gene (and HF gene) Fog2, which specifically suppress Gata-4 and Coup-TF2. However, when each miRNA is knocked out, we find that their targeting efficacies for Fog2 differ resulting in varying degrees of cardiac differentiation. Second, we apply and extend these developmental data to HF. Conditions such as coronary artery disease, high blood pressure, diabetes, etc., can cause cardiomyocytes to undergo hypertrophic changes that alter their structure and/or function ultimately resulting in HF. The current paradigm for treating HF is predominantly pharmacological intervention or surgery; however, with few exceptions, the condition usually worsens with time. Because the genes affected during development by miRNAs17, 20a, 93, 106a are also found mis-regulated during HF in adults, we have proposed a paradigm shift for treating HF from a drug-centric system to a molecular approach that would not simply treat symptoms but restore cardiac identity to affected cardiomyocytes. The approach utilizes the homeostatic duties of these four miRNAs to physically regulate genes that when mis-regulated demonstrably change the identity of cardiomyocytes.

P875/B896

Understanding the Role of the *Drosophila Melanogaster* Protein *Kismet*In Muscle Formation and Maintenance.

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Drosophila melanogaster serves as a useful tool for studying disease-causing homologs in humans. The *Drosophila* gene *Kismet* is homologous to the *CHD7* gene found in humans. CHD7 plays a role in chromatin remodeling, and mutations in this gene are known to cause a dominant genetic condition called CHARGE syndrome. CHARGE syndrome is a complex disorder that is characterized by colobomas of the eye structures, atresia, fused or missing kidneys, various heart defects, impaired cognitive and physical development, hearing loss, and other ear abnormalities. Individuals diagnosed with CHARGE may also exhibit swallowing difficulties, esophageal reflux, delayed fine and gross motor skills, and delayed walking. While some of these issues are neurological, not much is known about the associated abnormalities in muscle structure, though low muscle tone in skeletal and smooth muscles is apparent. The goal of this project is to analyze muscle structure and integrity in different mutants of *Kismet* in *Drosophila melanogaster*. Expression of the various *Kismet* mutants will be carried out using the Gal4-UAS expression system in the fruit fly. Studying the role of *Kismet* in muscle development in *Drosophila* may help in understanding how mutations in *CHD7* cause low muscle tone and affect early muscle development in humans.

P876/B897

Disruption of Inhibitory System for Autoubiquitination by an R441q Missense Mutation in the *Wwp1* Gene in Chicken Muscular Dystrophy.

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The HECT-type E3 ubiquitin protein ligases play a crucial role to maintain the cellular homeostasis by controlling the gene expression and protein turnover by ubiquitination of target molecules. The *WWP1* gene encodes a 130-kDa HECT-type E3 ubiquitin ligase (922 amino acids) composed of three functional domain regions, i.e., an N-terminal C2 domain for calcium-dependent phospholipid binding, a central region containing four tandem WW domains recognize substrates with the proline-rich peptide motifs, and a C-terminal HECT catalytic domain for ubiquitin transfer from E2 to substrates. An R441Q missense mutation in the molecule causes muscular dystrophy in NH-413 chicken. The amino-acid substitution is located in the center of the cluster of four WW domains (between WW2 and WW3 domains), speculating that it would lead to abnormal activity of WWP1 by the alteration of specific interaction to substrates. However, our analysis of NH-413 chicken clearly showed that the marked degradation and loss of the sarcolemmal localization of WWP1 in the skeletal muscles. This result suggested that the missense mutation gives rise to the loss of function of WWP1 by its degradation. To test the hypothesis, we generated mice having the same missense mutation as that found in NH-413 chicken WWP1 gene and examined the stability of WWP1 proteins in the knock-in (KI) mouse. Biochemical, histological, and cellular analyses clearly showed the loss of WWP1 protein in skeletal muscles of the KI mice, which would be caused by autoubiquitination. Moreover, in the skeletal muscle of the mice lacking WW2, the second WW domain, the remarkable protein reduction was also observed as is the case with NH-413 chicken skeletal muscle. These findings are consistent with the recent structural study of WWP2 E3 that the peptide linker tethering WW2 and WW3 can lock HECT catalytic domain in an inactive conformation and a WW2 domain can also lock the enzymatic activity by blocking the ubiquitin-binding exosite of the molecule. The high conservation of primary structures between the WWP2 and WWP1 proteins leads to the possibility that the WW2-WW3 peptide linker would be also a key regulatory element of HECT catalytic activity in the WWP1 molecule and the linker-mediated autoinhibition could be relieved by R441Q missense mutation.

P877/B898

Protein Networks of Heart Regeneration.

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The adult human heart is limited in its ability to regenerate after injury. Rather than producing new functional muscle tissue, the human heart forms scar tissue which often leads to heart arrhythmias and heart failures. Lower vertebrates like zebrafish have the ability to regenerate cardiac tissue after injury. Cardiomyocyte proliferation and activation of developmental programs have been shown to play key roles in zebrafish's ability to re-grow new tissue without scar formation. Transcriptional profiling and gene expression analyses in adult zebrafish have revealed profound changes in cardiomyocytes during heart regeneration. How these changes impact protein interacting networks in cardiomyocytes is unknown. Here we propose to capture and define the protein networks of different cellular compartments and regenerative factors in cardiomyocytes using BioID. This recently established

technique probes interacting networks of proteins by using a promiscuous Biotin ligase, BirA2, that attaches biotin to adjacent proteins. Biotinylated proteins are then isolated by streptavidin-based pull down and identified by mass spectrometry. Thus BioID allows capturing very transient protein interactions that might never be detected by standard methods. This study will define the functional networks of cardiomyocytes present during heart regeneration, and has the potential to reveal novel proteins that play key roles in cardiac repair after injury.

P878/B899

The Effects of AMPK Activators on Mitochondrial Turnover and Protein Synthesis in Developing Skeletal Muscle Cells in a High-Fat and High-Insulin Environment.

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AMP-activated protein kinase (AMPK) is a common target of diabetic therapies, and drugs that activate AMPK are increasingly applied to juveniles who are experiencing extensive muscle development. AMPK activation causes increased fatty acid oxidation, mitochondrial biogenesis, glucose uptake, and insulin sensitivity, but it also can limit protein turnover and muscle differentiation. Metformin, a member of the biguanide family, is an AMPK activator that, in muscle, targets the mitochondrial respiratory chain complex I. β-guanidinopropionic acid (β-GPA) is a creatine analog that mimics an energy stress within the cell, activating AMPK. The present study sought to assess the effects of high oleic acid (OA) and high insulin content on skeletal muscle cell differentiation, global protein synthesis, and mitochondrial turnover in the absence or presence of metformin and β-GPA. Cells treated with insulin produced significantly more myotubes per unit area over the 72-h treatment than all other treatment groups. Insulin treatment also elicited a significant increase in all markers of mitochondrial turnover (DRP1, SQSTM1, PGC1α, and OPA1) and global protein synthesis. The combined treatment of OA and insulin also elicited an increase in mitochondrial turnover markers, aside from OPA1, although the effect was smaller than for insulin alone, and there was no effect on global protein synthesis or myotube formation. In general, the addition of metformin or β-GPA reduced or eliminated the effect of insulin on mitochondrial turnover and protein synthesis. However, metformin groups that were cotreated with insulin or insulin + OA still displayed significant increases in markers of mitochondrial fission and mitophagy, but did not differ from controls in mitochondrial biogenesis, fusion, or global protein synthesis. Further, metformin + insulin resulted in high rates of myotube formation. These results are consistent with established effects of AMPK on protein synthesis, and growing evidence that mitochondrial turnover is compromised by AMPK-activating compounds.

P879/B900

Human Mesenchymal Stem Cells Primed with *Estradiol* Enhance*Angiogenesis* in Heart Tissue.

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Background/Aims: When detected early, heart disease, can be managed by life style changes or medical or surgical approaches. However, in advanced disease, heart transplantation remains the only viable option. Unfortunately, this option is constrained by the scarcity of gender-matched donors, and, also, highlights the importance of mesenchymal stem cells (MSCs) as a therapeutic tool for heart repair. However, inefficient incorporation, retention, and activity of MSCs in cardiac tissue remains an obstacle. Since surge in follicular estradiol (E2; μmolar-range) triggers tissue remodeling (e.g. ovulation) and E2

exerts beneficial actions on the cardiovascular system, we hypothesized that E2 may promote/improve MSC-mediated cardiac repair processes. **Methods:** Wharton's jelly (WJ)-derived MSCs were used, to assess the effects of E2 on their proliferation, directed-migration and engraftment in murine heart slices, *ex vivo* (measured using xCELLigence real-time cell-impedance system, DNA-quantification, and microscopy). Protein expression was assessed by Western blotting, ELISA/Luminex and proteomic analysis, whereas mRNA expression by qRT-PCR. **Results:** MSCs expressed estrogen receptors (ERs) -alpha and -beta. E2 promoted MSCs proliferation and up-regulated mRNA and protein expression of ER-alpha, ER-beta, extracellular matrix metalloproteinase inducer (EMMPRIN), and matrix metalloproteinase (MMP) -9, yet down-regulated MMP-2 expression. Moreover E2, up-regulated expression of vascular endothelial growth factor (VEGF)-A, vascular cell adhesion protein-1 (VCAM-1) and an giogenin (ANG), and stimulated nitric oxide (NO) production via ER. Proteomic analysis revealed that in MSCs, E2 up-regulated 47 proteins, down-regulated 7 proteins, and increased the expression of key biochemical components/pathways involved in tissue repair. In MSCs co-cultured with murine cardiac-slices, E2 significantly induced MSCs migration in an ER-dependent fashion (and preferentially via ER-alpha) and significantly increased the secretion of MMP-2, MMP-9, ANG and VEGF. In an in *vivo* matrigel assay, E2-treated MSCs increased angiogenesis and hemoglobin content. **Conclusion:** E2 facilitates the integration/engraftment of MSCs into heart slices by promoting MSC proliferation, migration and angiogenesis. These beneficial effects are mediated via increases in molecules/pathways involved in tissue remodeling and angiogenesis. We speculate that priming of MSCs with E2 may enhance their ability to repair/regenerate cardiac tissue in women.

P880/B901

Resolved Structural States of Calmodulin in Regulation of Skeletal Muscle Calcium Release.

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Calmodulin (CaM) is proposed to modulate skeletal muscle sarcoplasmic reticulum (SR) calcium release channel (ryanodine receptor, RyR1 isoform) activity in a conformation-dependent manner. However, the correlation between CaM structure and functional regulation of RyR in physiologically relevant conditions is largely unknown. We have used time-resolved fluorescence resonance energy transfer (TR-FRET) to study structural changes in CaM that may have a role in the regulation of RyR1. We covalently labeled each lobe of CaM (N and C) with fluorescent probes, and used intramolecular TR-FRET to assess interlobe distances when CaM is bound to RyR1 in SR membranes, purified RyR1, and a peptide corresponding to the CaM-binding domain of RyR (RyRp). TR-FRET resolved the equilibrium between two distinct structural states (conformations) of CaM, each characterized by an interlobe distance and width (disorder). In isolated CaM, at low Ca^{2+}, the two conformations of CaM are clearly resolved, centered at 5 nm (closed) and 7 nm (open). At high Ca^{2+}, disorder increases and the equilibrium shifts to favor the open conformation. In the presence of RyRp the resolution (order) of the two CaM conformations increases, with the closed conformation more compact and the major component. With CaM bound to full-length RyR1, either purified or in SR membranes, strikingly different results were obtained: (1) the two conformations are more clearly resolved and ordered, with the closed state more ordered than the open state. (2) the open state is the major component. (3) Ca^{2+} stabilized the closed conformation by a factor of two. We conclude that the Ca^{2+}-dependent structural distribution of CaM bound to RyR1 is distinct from that of CaM bound to RyRp. We propose that the function of RyR1 is tuned to the Ca^{2+}-dependent structural dynamics of bound CaM. These data suggest that the structure

of CaM when bound to the peptide is not representative of CaM binding to full-length RyR1. This striking result has implications for many other CaM binding partners that have been structurally characterized through peptide studies. Ongoing studies utilize purified RyR1 to directly measure CaM-RyR binding kinetics, the structural changes that contribute to regulation of the cardiac RyR isoform (RyR2), and how oxidation and disease-causing mutations in CaM affect the CaM-RyR interaction. This work was supported by NIH grants AG26160 (DDT), HL092097 (RLC) and 1F31AG052329-01A1 (MRM) and an American Heart Association Predoctoral Fellowship 15PRE25700131 (MRM).

P881/B902

***Dystrophin and Ensconsin* Have Opposing Roles in Regulating Nuclear Positioning.**

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Long-range movements of nuclei during skeletal muscle development is evolutionarily conserved suggesting that these movements are critical for proper muscle development. Consistent with this, one characteristic of poorly functioning muscle is disruption in the spacing of nuclei. Muscle cell structure is highly conserved between *Drosophila melanogaster and* mammalian muscle, making it an ideal model organism to understand the mechanisms that drive and the functions of nuclear positioning. We have shown that mutations in several genes associated with muscular dystrophies cause nuclear positioning defects in *Drosophila* that resemble their effects in patients. Specifically, *Dystrophin*, a Duchenne muscular dystrophy-linked gene, and *ensconsin*, a cofactor of the microtubule motor protein kinesin, are necessary for proper nuclear positioning. Both have been shown to bind microtubules in mammals, which could indicate they may regulate nuclear positioning via similar mechanisms. To determine if two genes are using the same pathway to coordinate position of nuclei, we conducted genetic interaction studies. An imals heterozygous for mutations in either gene individually displayed a minor disruption in the spacing of nuclei. Surprisingly, in animals that were heterozygous for a mutation in each gene, nuclear positioning was similar to that in wild type animals. These data suggest that *Dystrophin* and *ensconsin* work within the same genetic network, but that within this network they have opposing functions that must be properly balanced to efficiently position nuclei.

Scholarship of Diversity

P882/B1

Diversifying the STEM Doctoral Population: Time to Look Beyond the GRE and Revise Graduate Admissions Policies Diversifying the Stem Doctoral Population: Time to Look Beyond the Gre and Revise Graduate Admissions Policies.

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The selection of students for science, technology, engineering and mathematics (STEM) doctoral programs determines who will be leaders in research, higher education, and fields that underpin the U.S. economy. For decades, this selection process in most U.S. universities has relied heavily on the Graduate Record Examination (GRE), a test originally developed to "level the playing field" for applicants from variously ranked educational institutions. The merit of the GRE as a selection tool for graduate admissions has been long debated as well as its potential to disproportionally compromise admission of women and students historically underrepresented in STEM who score less well on the GRE than other groups. In the present study, we sought to determine whether GRE scores are predictive of PhD completion in a broader range of STEM fields and whether there are gender differences in the predictive abilities of the GRE. To obtain sufficient data for meaningful comparisons, we collected information on over 1800 students from four variously sized state flagship research universities that participate in the Northeast Alliance for Graduate Education and the Professoriate (NEAGEP). The Alliance is a long-standing collaboration originally funded by the National Science Foundation to diversify STEM PhD programs. We found that GRE Verbal (GRE V) and GRE Quantitative (GRE Q) scores were similar for women who completed STEM PhD degrees and those who left programs. Remarkably, GRE scores were significantly higher for men who left than counterparts who completed STEM PhD degrees. In fact, men in the lower quartiles of GRE V or Q scores finished degrees more often than those in the highest quartile. This pattern held for each of the four institutions in the study and for the cohort of male engineering students across institutions. GRE scores also failed to predict time to degree or to identify students who would leave during the first year of their programs. These findings show that GRE scores do not predict what are arguably the most important indices of preparedness for STEM PhD studies—persistence beyond the first year, time to degree or STEM PhD completion. In conclusion, we suggest it will be important to: (i) identify characteristics that motivated admissions committees to ignore the low GRE scores; (ii) determine whether these characteristics contributed to the success of the low-scoring men; (iii) develop more effective and inclusive graduate admissions strategies.

P883/B2

How Hhmi Is Encouraging Science Workforce Diversity through Institutional Change.

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Science education programs, including those funded by the Howard Hughes Medical Institute (HHMI), have historically promoted scientific workforce diversity mainly via student-centered interventions. While these programs can directly help student participants, they have generally not led to significant and lasting change required to close the gaps between those who become scientists and those that are excluded from science. We believe that much of the responsibility for advancing diversity does not rest

on students but on institutions in which students find themselves. Therefore, the HHMI Science Education Undergraduate and Graduate Programs (UGP, https://www.hhmi.org/science-education/programs) has shifted away from funding programs that solely focus on "fixing the students" to those that address institutional barriers for participation of persons who belong to groups historically excluded from science. We pursue a systems approach aimed at establishing a more inclusive scientific environment. Our hypothesis is that, by focusing on learning environment and institutional culture, improvement in diversity and inclusion will be substantial and sustained. This approach informs all UGP programs, including: the Inclusive Excellence initiative, which seeks to increase the capacity for inclusion on each grantee campus through faculty development, curriculum revision, and changes in policies and practices; the Gilliam Graduate Fellowships Program, which engages the dissertation advisers of the Gilliam Fellows in a year-long series of activities that provide culturally-responsive mentor training; the HHMI Professors initiative, which empowers accomplished scientist faculty to explore critical issues in education and transform the climate for education at research universities; and the HHMI Science Education Alliance, whose PHAGES course is an inclusive course-based research and educational community (iREC), enabling beginning undergraduates from all backgrounds and all institution types to engage in authentic research. We recognize our responsibility to learn from the grantees and to continually refine frameworks that can inform future efforts. Applying theory of change practices, we are actively examining our assumptions about these initiatives and strive to foster a dynamic stance to promote our aspirational goal: inclusion is foundational to excellence in science.

P884/B3

Scientific Societies Join Forces to Amplify Effectiveness of Stem Workforce Diversification Programming.

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Despite increasing awareness of the benefits that come with having a diverse STEM workforce, efforts to increase the number of URM scientists have met with limited success. In professional scientific societies, diversity-focused committees continue to play essential roles in the efforts of scientific societies to foster inclusion and facilitate the professional development of underrepresented minorities (URMs) in their respective scientific disciplines. Until recently, the efforts of these committees remained independent and disconnected from one another. Funding from the National Science Foundation has allowed several of these committees to come together and form the **A**lliance to **C**atalyze **C**hange for **E**quity in **S**TEM **S**uccess, herein referred to as ACCESS. The overall goal of this meta-organization is to serve as a community in which diversity-focused committees can interact, synergize, and have a unified voice, enabling them to better address the needs and concerns of URMs in STEM disciplines based on their collective experiences. To accomplish this, ACCESS meets regularly to share data and best practices in programmatic assessment and structures. As an example of our work, we compare and contrast the broad range of ways in which scientific societies implement and assess their travel award programs for

URMs. We also report a set of recommendations, including both short- and long-term assessment of outcomes in populations of interest and specialized programmatic activities coupled to travel award programs. ACCESS plans to launch a website in 2020 to broadly disseminate ways in which societies are implementing STEM workforce diversification programs, such as travel awards. Our ultimate goal is to broaden ACCESS to encompass additional societies, including those wanting to develop programs for diversity and inclusion and those with existing programs.

P885/B4

Mental Health and Wellness in Stem: Facilitating an Open and Necessary Discussion About Our Community and Our Identity.

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Graduate student and postdoctoral mental health and wellbeing has only recently emerged as a topic of serious discussion within the STEM community. Surveys of STEM trainees have revealed that a significant number of members of our research community face mental health and wellness challenges at some point throughout their training careers. Furthermore, seeking help for mental wellness can be a major challenge during STEM training, and an even larger challenge for underrepresented scholars. Our goal was to develop and facilitate a workshop that not only discussed the current evidence that mental health and wellness challenges are common in our STEM research community, but we also wanted to invite trainees to join a “brave space” in which to consider resources and strategies to acknowledge and seek support for their mental health. To this end, we designed a workshop that focused on how our community of STEM researchers contribute to mental health and wellbeing. Using a case study approach, we ask participants to consider not just the trainee facing mental health challenges, but also to consider the perspective of cohort/lab mates, and even the perspective of the advisors/mentors. We want to make the point that if our career plans are to remain in academia, we may participate as the trainee, the labmate, and the mentor at some point in regards to the mental health of trainees in the community. We argue that understanding how each member of the STEM training community can impact a mentee’s experience is crucial. Moreover, we discussed how our personal identities impact our experiences with mental health, and how the identities of those in our community may impact how we interact with them or share with them during times of mental health struggles. In the last year we have presented this workshop at the SACNAS National Conference, at the ABRCMS National Conference, at UC San Francisco, and at UC Berkeley. We continue to revise and improve the workshop with each presentation and look forward to facilitating these important conversations about mental health and wellness with STEM trainees, faculty, and administrative staff.

P886/B5

The Impact of a Short Summer Bridge Program on Success and Retention of First Generation Biology Majors at a Residential Liberal Arts College.

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To address the shortage of skilled STEM professionals in the US, as well as the need for increased representation in the STEM workforce, many colleges and universities have developed summer bridge programs designed to improve the persistence of first-generation, low income, and/or underrepresented students in STEM majors. At Susquehanna University, a small (2,300 total students with about 300 life science majors) undergraduate institution in rural Pennsylvania, we have conducted

summer bridge programs for the past 8 years, initially supported by NSF funding and more recently by the university. Each year, we invite approximately 10 students to attend. Invitations are extended primarily to first-generation students with high financial need. This targeted population typically includes significant racial diversity. These students spend a week on campus the summer before matriculating, learning about research opportunities, working with science faculty, and gaining an introduction to campus resources. Equally important, it allows them an opportunity to develop a social network of peers prior to arriving on campus in the fall. The program is free of charge to the students and operates at a modest cost to the institution (approximately $10,000 per summer). We have previously shown that students who participated in the first 4 summer bridge programs graduated with science degrees at a significantly higher rate than a matched control group. In this study, we analyze more specifically the impact of the summer bridge program on a variety of success markers as well as a self-reported sense of belonging as a science major. We found that sex and race significantly affected students' level of agreement with the statement "I can do well in the sciences at Susquehanna". While participation in a pre-orientation program did not significantly affect students' level of agreement with the statement, there was a significant interaction between race and participation in a pre-orientation program. We found white students had higher first semester GPAs (3.24+/-0.10 v. 2.83 +/-0.14) and second semester GPAs (3.29+/-0.13 v. 2.75+/-0.17) than students of color. However, when we included the number of high school science classes as a covariate in the model, we found that race no longer has a significant effect on first semester GPA. White students reported taking significantly more science classes in high school. In summary, there are multiple ways in which student identity, background, and participation in a short summer bridge program impacts student success and sense of belongingness for under-represented biology students.

P887/B6

Insights from a Survey-based an alysis of the Academic Job Market.

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Many postdoctoral fellows in the STEM fields enter the academic job market with little knowledge of the process and expectations, and without any means to assess their qualifications relative to the general applicant pool. Demystifying this process is critical, as there is little information publicly available. In this work, we provide insight into the process of academic job searches by gathering data to establish background metrics for typical faculty job applicants, and further correlate these metrics with job search outcomes. We analyzed 317 responses to an anonymous survey for faculty job applicants from the May 2018 - May 2019 market cycle. Responses were about evenly split by gender, largely North American-centric and life science focused, and highly successful with 58% of applicants receiving at least one offer. Traditional metrics (funding, publications, etc.) of a positive research track record above a certain threshold of qualifications were unable to completely differentiate applicants that did and did not receive a job offer. Our findings suggest that there is no single clear path to a faculty job offer and that perhaps criteria not captured by our survey may also influence landing a faculty position above a certain

threshold of qualification. Furthermore, our survey did capture applicants' perception of the faculty job application process as unnecessarily stressful, time-consuming, and largely lacking in feedback, irrespective of a successful outcome. We hope that this study will provide an avenue for better data-driven decision making by the applicants and search committees, better evidence-based mentorship practices by principal investigators, and improved hiring practices by institutions.

P888/B7

The ASCB MAC Faculty Research and Educational Development (FRED) Program Has Resulted in Program Participants/Mentees Who Successfully Compete for Grant Support.

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Access to national funding is generally required for career advancement and promotion/tenure and was identified as a barrier for junior faculty members in a recent study. The Minorities Affairs Committee (MAC) of the ASCB has a long history of providing career development opportunities for minority cell biologists. In 2013, a team of MAC members applied to the NSF for support for the Faculty Research and Education Development (FRED) Program, an intervention to promote grant funding success of minority senior postdoctoral fellows and junior faculty members and junior faculty members at MSIs. The year-long FRED Program activities kick off with a three-day summer workshop in which the mentees learn about NIH and NSF funding opportunities and hear from near peers and MAC members about the tenure process and collaboration establishment. A major component of the workshop is the one-on-one mentee-mentor meeting time to work on the specific aims or summary section of the proposal. Each of the mentees is required to submit a proposal for the mock review panel/study section held during the ASCB annual meeting in December. The mentees and mentors serve as reviewers during this mock session. Also, each pair of mentee and mentor engage in reciprocal visits to each other's institutions. This is critical for the mentors from research-intensive institutions to appreciate the research environment of the faculty at MSIs and more teaching-intensive institutions. The FRED Program has had 5 cohorts of mentee and mentor pairs. The 6th cohort will finish the Program in June 2020. The mentees have been a diverse group of individuals - 42% are Hispanic, and 20% are African-American. Over 60% are women. These mentees reported promotions, publications, collaborations, and other significant events that came about in part because of their involvement in the Program. While the national funding rates at the NSF and NIH are below 10%, 19 (53%) of the FRED mentees have been successful in attaining funding with 28 total proposals funded. Thus, the FRED Program model has been an effective approach for junior faculty to compete successfully for grants from NIH, NSF, and other federal agencies, as well as from private-sector funding entities. This work was supported by NSF Award 1340395.

Science Education: Innovations in Teaching and Learning

P889/B9

Share Your Expertise and Enthusiasm with Teachers: Organize a Workshop to Build Inexpensive Microscopes for Classrooms.

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To get low cost microscopes in front of kids and teachers, I've been running microscope-building workshops for elementary school teachers. I use Kenji Yoshino's design, which he described in an Instructable entitled "$10 Smartphone to digital microscope conversion!" This is a simple and clever design for a low cost, DIY microscope that takes advantage of ubiquitous smartphone cameras. The microscopes are mostly transparent, making their inner workings obvious to teachers and kids. An d all of the parts are readily available from hardware stores or online, so teachers or kids can make more anytime if they wish. Based on feedback from teachers, I enlarged Kenji's design to accommodate iPads and other tablets, which are available in many elementary schools, and I've made other minor improvements. Teachers have reported back that groups of kids interact with each other around each microscope, looking at the smartphone or tablet screen together — a big improvement over microscopes in which kids might see only their own eyelashes, and in which only each kid would know what they saw. For the workshops, I prepare enough materials for each teacher to build their own microscope to keep in their classroom. A workshop with about a dozen teachers can equip a school with a complete set to move from classroom to classroom. Most of the workshop last 90 minutes, during which three things happen: (1) the teachers assemble the microscopes, (2) they develop expertise using the microscopes as they take pictures of things they brought with them, and they collect images to share (see image galleries at www.diymicroscopes.org), and (3) teachers brainstorm about how they'll use the microscopes to best match their curriculum needs and kids' interests. My current work includes bringing workshops to high poverty school districts in North Carolina and to statewide teachers conferences, and setting up to assess outcomes. At this poster there will be some of the microscopes for anyone to try, and I'll share a protocol that you can use to get started running your own workshops with teachers at local schools. An d I'll be happy to talk with people about collaborating or helping you get started running similar workshops on your own.

P890/B10

Building a Successful High School Outreach Project with Undergraduate Mentors: the Prince Edward County Environmental Molecular Biology Institute (PECEMBI).

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With financial support from an ASCB Public Engagement Grant funded by the Science Sandbox, an initiative of the Simons Foundation, we launched the Prince Edward County Environmental Molecular Biology Institute (PECEMBI), an outreach program connecting the students of Hampden-Sydney College and Longwood University with the students of Prince Edward County High School, Virginia. Prince Edward County is a rural, underserved school system in central Virginia with a significant population of students from groups traditionally underrepresented in the STEM disciplines. In the fall semester, undergraduates from Hampden-Sydney and Longwood visited the high school each week and worked with biology students on a long-term project that explored the microbiome of student-selected locales around the school and introduced students to microbiology and molecular biology techniques. The spring semester featured work on examining the microbial contents and the quality of the watershed around the high school to consider issues more directly related to environmental biology. Both semester's activities culminated with a public presentation in coordination with Longwood's student research symposia. While Hampden-Sydney and Longwood faculty were present at each session, the undergraduates led all activities and discussions with the high school students as much as possible.

Assessment of the program conducted via surveys administered to high school students and teachers as well as Hampden-Sydney and Longwood undergraduates and faculty revealed high levels of enthusiasm and satisfaction among all participating parties in the program. While the high school students especially enjoyed the opportunity to participate in an authentic research experience and to interact with the undergraduate students, they expressed their hope that in future iterations of the program that the specific undergraduates working with them would not change from week to week. Likewise, both the high school and undergraduate faculty expressed praise for the overall work of the undergraduates but saw value to place more training emphasis in future iterations on the cultural aspects of successfully mentoring high school students. Overall, PECEMBI gives high school students insight into how college level research is done, how to summarize and present their research at a university poster session, and how to respond to peer criticism. It is PECEMBI's goal to supplement the scientific educations of the high school students in Hampden-Sydney's and Longwood's backyard and better prepare them for potential careers in science.

P891/B11

Six New York City (nyc) Medical Schools Will Offer Two Summer-long, Eight Weeks/summer, Professional Development (pd) Programs for New York City'S Secondary Science Teachers Beginning in Summer 2021.

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The U.S.'s current biomedical research workforce does not mirror the demographics of its population, seriously limiting our nation's scientific enterprise. Research shows that workforce diversity has beneficial outcomes including increased productivity, higher quality, and higher impact work (Valantine, H.A. An d Collins, F. S. *PNAS U.S.A.*112, 12240-12242, 2015). However, to increase diversity of the biomedical workforce, we must increase the number of underrepresented (UR) students entering STEM fields. UR students show strong interest in STEM fields upon matriculation into college, but 50-70% of them drop out before graduation (Meyers L.C., *PLoS ONE*13(1), 2018). Of all U.S. bachelor degree recipients, only ~21,000 Black and ~26,000 Hispanic students received STEM degrees, compared to ~195,000 White and ~39,000 Asian students (https://nces.ed.gov/pubs2016/2016007.pdf). This deficit in STEM education of UR students is due, in part, to inadequacies of secondary science teacher preparation in teaching inquiry-based science. The educational skills of most science teachers plateau 3-5 years after their entry into teaching. Columbia University's Summer Research Program (CUSRP) for Secondary Science Teachers was designed to address these deficits. Outcomes studies show that following teacher completion of Columbia's two summer-long, eight week/summer program, 10% more students of participating teachers pass New York State Regents science exams than students in classes taught by non-participating teachers in the same school at the same time. Participating teachers are retained in education at an ~3.5-fold higher rate than non-participating teachers. As such, New York City's (NYC) Department of Education saves educational costs for courses and exams students do not have to repeat and teachers it does not have to replace (*Science*326, 440-442, 2009). The program, its graduates, and their students have been recognized by 75 national, state and local educational awards since 2003 and has been replicated successfully at multiple sites, both in and outside of New York State. The program's success has prompted AMSNY, whose mission is to increase diversity of the biomedical and physician workforce, to engage a consortium of six of its member medical schools, to jointly implement CUSRP-like secondary science teacher professional development (PD) programs in NYC. These PD programs will

result in the further professional growth of experienced teachers, higher science teacher retention rates, elevation in the quality of STEM education in NYC's schools and a significant increase in the number of UR students prepared for post-secondary STEM education and careers.

P892/B12

Benefits of Collaborative Learning in Undergraduate Neuroscience Education.

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Collaborative learning is an evidence-based instructional strategy that facilitates rich discussions between students to deepen student engagement and learning. This pedagogical approach increases learning gains and reduces failure rates among undergraduate students in STEM courses. In addition, student participation, motivation, and self-efficacy can all be increased with these structured learning environments. One such structured, active learning environment is Team-Based Learning (TBL). In TBL, students spend the majority of classroom time applying course content, analyzing data, synthesizing new ideas, and evaluating hypotheses. Importantly, previous studies have shown that learning outcomes and student course satisfaction are higher in courses that use TBL, compared to lecture-based classrooms. To better understand how TBL improves student learning and course satisfaction, I collected end of semester course evaluations and measured student-perceived classroom dynamics, as well as learning of lower and higher order levels of Bloom's taxonomy. My results suggest that implementation of TBL in an undergraduate neuroscience classroom improves student-perceived learning in both lower order (gaining knowledge, understanding concepts) and higher order (learning to apply and synthesize) levels of Bloom's taxonomy. These increases are consistent with the strong emphasis placed on application and synthesis within a TBL classroom.

P893/B13

Using Yeast Two-hybrid in Introductory Biology to Reinforce Molecular Biology Skills and Concepts.

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Course-based undergraduate research experiences (CUREs) expose a broad audience to authentic research questions and permit a professor to continue their own studies in the context of a high teaching load. As part of a second semester introductory biology course that emphasized genetics, students conducted an eight-week yeast two-hybrid screen to identify novel interactors of a zebrafish mitochondrial protein using a zebrafish cDNA library. In the context of yeast two-hybrid, students learned a variety of basic laboratory skills including DNA isolation from both yeast and bacteria; restriction digests, plasmid mapping, and agarose gel electrophoresis; bacterial transformation and yeast mating; and in *silico* methods analyzing raw DNA sequences and mining a genome database. Throughout this process, lecture concepts regarding gene structure, transcription, and translation were reinforced. Students identified many potential interactors, which they then researched through primary literature and protein databases to evaluate as bonafide candidates or false positives based on subcellular localization and known cellular function. After these informed predictions were made, the students then did pairwise validation matings to refute or affirm their hypotheses. Finally, they described their project and proposed alternative methods to identify protein-protein interactions in a cumulative research paper. Importantly, this large-scale screen served not only as a classroom tool, but also provided a new trajectory for later independent projects in the research laboratory.

P894/B14

Using Iteration and Relevancy to Scale Authentic Research Experiences for All Introductory Biology Students.

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The seminal educational report *Vision and Change*called upon scientists and educators to provide all their students with authentic research experiences in teaching laboratories. Course-based undergraduate research experiences (CUREs) scale research experiences for many students in a teaching laboratory setting. However, implementing CUREs in introductory classes is challenging and often requires students to opt-in to the experience through specialized first-year programs. Here we describe a simple CURE innovation that uses iteration and sustains relevance throughout the semester to provide students with a genuine research experience in their introductory cell and molecular laboratory course. As measured by the Laboratory Course Assessment Survey (LCAS; n = 63), students perceived this iterative lab course similar to how students perceived their specialized CURE courses. Over the semester, students exhibited marked gains in scientific self-efficacy. Additionally, students reported cognitive project ownership similar to students in research laboratory courses. Thus, students in this minimally redesigned introductory laboratory reported many of the gains often restricted to upper-level or specialized research-based laboratory courses. We offer a low-cost model for introductory laboratory courses that easily scales within traditional time allocations as a powerful intervention at no additional costs to institutions.

P895/B15

Fibroblast Spheroids and Other Models of Wound Healing and Infectivity.

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The goal of our research is to create a functional in *vitro* 3D model of skin for the purpose of experimentation. This project is being carried out in Montgomery College as part of a semester-long Introduction to Scientific Research class, taught to second year students. Students start using NIH 3T3 fibroblasts in two dimensional monolayers (2D), then transition to the use of spheroids, in-vitro three-dimensional models (3D) while conducting novel independent mentored research. We optimized parameters of spheroid growth, such as time, size and the number of cells in culture. In parallel, we optimized the MTT assay to see if it correlated with spheroid growth and our results agreed with published studies. We obtained reproducible decreases in spheroid viability using hydrogen peroxide to induce cellular damage and we obtained similar decreases when we used *Staphylococcus aureus* lipoteichoic acid (LTA) to induce a stress response associated with infection. An other substance tested was Manuka Honey, which has been shown to be effective in wound healing without scarring and possesses antimicrobial activity. In this case, we observed a reproducible increase in cell viability which suggested improved spheroid health. We have modified wound healing assays by using a combination of monolayer and 3D systems. Our results suggest that these hybrid spheroid models may be useful to identify natural compounds with relevance to wound healing.

P896/B16

Building Better Beer through CUREs: Undergraduate Classroom/brewery Collaborative Molecular and Cellular Biology Research.

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It is established that undergraduates benefit greatly from the introduction of authentic research experiences into life science coursework; however, such experiences can be difficult to engineer in a meaningful way, especially at smaller institutions where instructors must balance high levels of teaching with scholarly activity. An other issue arises with an enthusiasm gap observed by many students when the research opportunities presented in classes do not have medical or clinical relevance. To address both of these issues and to generate an exciting authentic research experience for our molecular and cellular biology classes, we teamed up with Third Street Brewing, a microbrewery based in Farmville, Virginia, to develop a semester-long course-based undergraduate research experience (CURE). In this CURE, students were tasked with "improving" budding yeast (*Saccharomyces cerevisiae*) strains according to the specifications of the brewery. For the pilot run of the project, the brewery specifically requested modifications that would alter flocculation tendencies of the yeast cells or the production of specific chemical byproducts during fermentation. Students were tasked with bioinformatics analyses to find target genes to manipulate in support of the brewer's goals and to develop research proposals. Over the course of the semester, students did gene deletions, site-directed mutageneses, gene induction experiments, and other molecular biology manipulations that could be tested for the brewer's specifications. While the semester did not provide enough time for most groups to bring an altered strain to completion, the experimental progress laid the groundwork for future students to complete the strains in either independent research or CURE settings. Assessment of the CURE through post-course surveys of students revealed that the project was successful in generating enthusiasm for learning molecular biology techniques and cell biology processes through CURE-based work on a project with obvious practical applications. The assessments also revealed a discomfort with the level of freedom provided throughout the project and the lack of specific instructions for completion of the project, suggesting that future iterations of the CURE may benefit from the addition of guidelines that provide more structure to the project while preserving the independent spirit of the CURE. Overall, this CURE showed the utility of working with businesses and organizations in the local community to develop research opportunities for undergraduates that can generate enthusiasm through the potential for discernable practical benefits. The use of budding yeast in the context of brewing also provided a CURE model that is accessible to institutions of all types and infrastructure levels.

P897/B17

Factors That Predict Life Sciences Student Persistence in Undergraduate Research Experiences.

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Undergraduate research experiences (UREs) have the potential to benefit undergraduates and longer UREs have been shown to lead to greater benefits for students. However, no studies have examined what causes students to stay in or consider leaving their UREs. In this study, we examined what factors cause students to stay in their UREs, what factors cause students to consider leaving their UREs, and what factors cause students to leave their UREs. We sampled from 25 research-intensive (R1) public universities across the United States and surveyed 768 life sciences undergraduates who were currently

participating in or had previously participated in a URE. Students answered closed-ended and open-ended questions about factors that they perceived influenced their persistence in UREs. We used logistic regression to explore to what extent student demographics predicted what factors influenced students to stay in or consider leaving their UREs. We applied open-coding methods to probe the student-reported reasons why students chose to stay in and leave their UREs. Fifty percent of survey respondents considered leaving their URE, and 53.1% of those students actually left their URE. Students who reported having a positive lab environment and students who indicated enjoying their everyday research tasks were more likely to not consider leaving their UREs. In contrast, students who reported a negative lab environment or that they were not gaining important knowledge or skills were more likely to leave their UREs. Further, we identified that gender, race/ethnicity, college generation status, and GPA predicted which factors influenced students' decisions to persist in their UREs. This research provides important insight into how research mentors can create UREs that undergraduates are willing and able to participate in for as long as possible.

P898/B18

The Impact of Student Research an xiety on Undergraduate Intention to Pursue a Scientific Research Career.

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Participating in undergraduate research is one of the most lucrative activities that a biology student can engage in because of the wide array of benefits that research can provide. Yet, while undergraduate researchers are more likely to persist in science than their peers who do not participate in research, there are still many undergraduate researchers who choose not to pursue a research career. In this study, we explored to what extent research anxiety influences students' intentions to pursue a research career. Specifically, we explored whether demographics predicted student research anxiety and their intention to pursue a research career. We were also interested in whether students' perceived difficulty of their research predicted research anxiety. We sampled from 25 public R1 universities and surveyed 768 biology majors who had participated in a research experience during the academic year. On the survey, we measured student research anxiety, research difficulty, intent to pursue a research career, and demographics. Students also answered open-ended questions about what elements of their research experience affected their anxiety. Using structural equation modeling, and controlling for students' intentions to pursue a science-related research career before they entered their undergraduate research experience, we explored the relationships between student demographics, research anxiety, research difficulty and intent to pursue a research career. We found that females were more likely to have higher research anxiety than males. Additionally, compared to students with low research anxiety, students with high research anxiety were less likely to report intentions to pursue a science-related research career. We also identified that the more difficult a student perceived their research, the more likely they were to express high research anxiety. Finally, using open-coding methods, we coded students' responses to what increased and decreased their research-anxiety. This work identified a novel factor, research anxiety, that influences students' intentions to pursue a science-related research career and identified ways in which research mentors can work to decrease student research-anxiety with the intent to create a more diverse and inclusive scientific community.

P899/B19

The Genomics Education Partnership: a Community of Practice That Enhances Research Opportunities for Students and Faculty at Diverse Institutions.

M. Van Stry; Lane College, Jackson, TN.

Since 2006, the Genomics Education Partnership (GEP) has incorporated authentic genomics research experiences into the undergraduate curriculum, introducing thousands of students to eukaryotic gene structure, comparative genomics, and the evolution of *Drosophila*. Our 100+ participating institutions include community colleges, primarily undergraduate institutions, minority-serving institutions (MSIs), historically black colleges and universities, and research-intensive PhD-granting institutions. For many faculty and their students, the accessible, immersive curriculum and custom bioinformatics tools represent a unique opportunity to participate in research. GEP faculty benefit from membership in a national network of like-minded colleagues and professional development opportunities that include training, research, and publication in peer-reviewed journals. Students who resolve sequencing problems and generate high-quality gene models for GEP analyses are eligible to be co-authors on the scientific publications based on their contributions. GEP has partnered with Galaxy to develop G-OnRamp, an open-source platform for constructing UCSC Assembly Hubs and JBrowse/Apollo genome browsers for eukaryotic genomes, thereby enabling crowd-sourced gene annotation using the GEP curriculum. G-OnRamp allows for more varied research projects to be incorporated into the GEP portfolio, including a current investigation of venom evolution in parasitoid wasps and an investigation of the evolution of insulin pathway genes across 27 *Drosophila* genomes. Our ongoing work in science education finds that a bioinformatics CURE fosters “formative frustration” where students can safely fail in their original analysis, adjust, recover, and succeed. This iterative process allows students to gain deeper insight into annotation and occurs quickly within an inexpensive, online framework. The GEP is actively recruiting additional faculty members, especially from community colleges and MSIs to participate in regional and national professional development to use the GEP curriculum in their classrooms. Further, the GEP is seeking science partners who can collaborate with GEP members to fund and develop additional projects, and science education partners to assist with curriculum development and assessments. Supported by NSF IUSE-1431407 to SCRE, NSF IUSE-1915544 to LKR, and NIH IPERT-1R25GM130517-01 to LKR.

P900/B20

Artlab Vanderbilt: Bringing Together Artists, Scientists, and Designers to Facilitate Science Outreach and Communication.

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ArtLab is a campus wide organization exploring the intersection of the arts and the sciences at Vanderbilt University. The goal of ArtLab is to promote discussion of scientific research in an approachable setting that allows people of multiple disciplines to get involved. ArtLab has hosted multiple exhibitions (Ex. ArtLab: Exploring the intersection of art and science, ArtLab Opener 2017, ArtLab at Vanderbilt) that included artistic work by undergraduates, staff, and faculty from across the University community. By forming a campus-wide network with key interest in building an art-science community, we have collected both in-person and online survey data that explores the interest in the program since 2017. The collected data comes from content presented at all exhibitions. We have found an increased interest in these science exhibitions from a wide range of disciplines, supporting our goal.

Most importantly, the exhibitions provided an outlet for both scientists and non-scientists to come together and discuss current topics of research. Surveys provided insight into how attending these events enhanced interest in scientific research discussion. Here we present these findings and suggest possible next steps to perpetuate transdisciplinary interactions across campus and the community focused on public engagement and education of the basic sciences.

P901/B21

The Genomics Education Alliance.

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The Genomics Education Alliance represents a group of life science educators with experience in engaging students in genomics-based Classroom-based Undergraduate Research Experiences (CUREs). Because we are convinced that CUREs are effective for students to learn both key concepts and the practice of science, we have come together to identify and overcome common barriers to put such experiences within the reach of all life science faculty and students. To achieve this goal, the alliance will curate curriculum and assessment materials, and make them freely available for use on the CyVerse platform. Currently supported by a National Science Foundation Research Coordination Network grant, we are now piloting a set of materials for use by educators in their classrooms in three areas: lessons on examining gene sequence similarities using BLAST, understanding gene structure by using a eukaryotic genome browser, and investigating gene expression by using basic tools for RNA-seq analysis. Eventually, we will curate a wide variety of materials both from our existing genomics CUREs and new resources we create, and make available the appropriate compute resources necessary for use in the classroom. Ultimately, we aim to facilitate efforts by faculty who build their own genomics CURE using our optimized resources. We are currently recruiting faculty to test our pilot resources; let us know if you're interested. Supported by NSF RCN-UBE grant # DBI 1827130

P902/B22

An Integrated Undergraduate Steam Course: the Art and Science of Cell Death.

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This fully integrated laboratory-and studio-based course enabled students to visualize apoptosis, design experiments around this basic cell biology process, and then use those experimental data to generate two art pieces. After completing this interdisciplinary course, students fulfilled both their art and science general education requirements. Through short lectures and demonstrations, a mix of thirteen biology and non-science majors were introduced to both the intrinsic pathway of apoptosis and the principles of art and design. Students first learned basic cell culture techniques through a guided experiment expressing Bax-GFP in Omi-RFP HeLa cells. Images were then captured on a confocal microscope and used to thematically inform an exquisite corpse drawing that was digitally rendered using a 3D computer-aided design (CAD) program to create a computer numerical control (CNC) milled wood panel. Following this, students in small groups applied their laboratory experience to design and test their own apoptotic protein using genetic engineering and synthetic biology techniques. Here, we present data generated by two student groups that fused a CARD or a DED domain to GFP to accelerate cell death, as

well as another group that engineered caspase cleavage sites into a dual fluorescent protein to serve as an indicator of apoptosis. Finally, using their personally collected confocal images, students digitally designed and fabricated a 3D printed sculptural form. In the context of this class, students practiced critical thinking and problem-solving skills utilizing both hands-on, novel experiments and computer-aided design.

P903/B23

A Data an alysis and Literature Intensive Undergraduate Course That Positively Impacts Student Ability and Confidence in Scientific Critical Thinking Skills and Increases Post-graduation Success.

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Bio 404: Nuclear Structure and Function is an advanced cell and molecular biology course taken by junior and senior undergraduates at Westminster College (New Wilmington, PA). While the content of the course focuses on architectural aspects of nuclear cell biology and molecular understanding of gene expression and genome structure, the major objective of the course is to broaden students' skills in content comprehension, data-analysis, modeling, and productive scientific discussion using literature. The design builds upon existing models for using data analysis or individual journal club sessions in order to maximize student gains in ability, confidence, and postgraduate preparation. The four course units (Nuclear Periphery, Nuclear Pore, Chromatin and Nuclear Bodies), are divided into multiple, one-week modules consisting of three one-hour sessions. 1) Secondary article based lecture, introducing a theme. 2) "Work it out Wednesday" where students work in groups of 3-4 answering questions to analyze data and interpret results in order to develop a biological model that links back to lecture. 3) Student-led journal club discussing a theme correlated article. Course evaluations from the 2016 offering indicated that students thought the course was well organized, was a valuable learning experience and that the assignments had instructional value; moreover, exam and course grades in 2016 improved over the 2014 offering, which was structured in a more traditional lecture style. As a result of the positive outcomes with the change in course format a more formalized analysis of student gains and perceptions of the course was performed in the 2018 offering, together with a survey of the 2016 alumni. Student gains in ability and confidence with critical thinking skills were assessed and compared to the pre-requisite course and alumni. Greater gains across several metrics occurred in the advanced course, with students closing the pre-course gap between ability and confidence. Alumni retained gains and reported advantages over peers in postgraduate programs. This presentation will consist of an example of a course module, data from this initial study and ideas for application of the method to a variety of content areas and course sizes.

P904/B24

Improving Awareness About Scientific Rigor and Reproducibility through NIDDK Information Network (dkNET) Tools and Training Resources.

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When results of biomedical experiments can not be reproduced, the conclusions are then questioned. Poorly characterized antibodies and cell lines contribute to this problem, as do incomplete reports of materials used, lack of fully documented experimental designs and statistical approaches and the lack of published data. In 2016, NIH announced new guidelines for rigor and reproducibility in grant

applications. The NIDDK Information Network (dkNET; https://dknet.org) is an open community resource portal for researchers in diabetes, digestive, endocrine, metabolic, kidney, and urologic diseases [1]. To enhance the awareness about scientific rigor and reproducibility, dkNET developed tools, resources, and training modules. Based on Research Resource Identifiers (RRIDs), unique identifiers for research resources [2], we developed tools such as Resource Reports and Authentication Reports to assist researchers in the identification and authentication of cell lines and antibodies. These reports provide alerts when the resources have problems and also provide validation information, if available. dkNET also provides informational pages about Rigor and Reproducibility, RRIDs, data management, and suggested data repositories. Building on this material, dkNET has been hosting webinars around these concepts of reproducibility and related dkNET tools. All these recordings are available on dkNET's YouTube channel (https://bit.ly/30Ngyzt). This year we launched the dkNET Summer of Data Student Internship Program to provide students with an opportunity to utilize dkNET tools and resources in their research projects and to learn the basics of rigor, reproducibility and good data management for FAIR (Findable, Accessible, Interoperable, Reusable) data. Students received hands-on training on how to use dkNET to find and evaluate research resources, how to use online data and tools to develop hypotheses to enhance their projects as well as learning the best practices in data management for robust and reproducible research. A course survey indicated that students increased their familiarity with the tools and concepts taught in the course, and are more comfortable with performing reproducible research. In summary, dkNET provides useful online resources and training modules to assist researchers in improving scientific rigor and reproducibility. Graduate program educators can integrate dkNET resources into the research conduct curriculum. References: (1) Whetzel PL et al., PLoS One. 2015; 10(9):e0136206. (2) Bandrowski AE et al., Neuron. 2016; 90(3):434-6. Source of Support: NIH NIDDK Grant U24DK097771

P905/B25

It's Better Together: Adopting Collaborative Learning Techniques to a Community College Cell Biology Course.

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Biology I: Molecules & Cells (BIOL 110) is a gateway course, a prerequisite for science and allied health majors at Community College of Baltimore County (CCBC). It often becomes a "roadblock course" for many of the CCBC students aspiring for a career in STEM or healthcare. The course has a high dropout and failure rate, and while many students repeat the course, the "repeaters" are often less successful at passing the course than the "first time takers". The standard format of the course includes traditional lecture and laboratory. Student participation in the lecture classes is limited to note taking, and clicker questions. Such approach often results in the psychological isolation and alienation of the community college students, who have fewer opportunities for socialization compared to their peers attending a four-year institution. Lack of social interaction negatively impacts performance and persistence in the major. Multiple studies (Johnson et al, 2014; Smith et al, 2009) indicated that collaborative learning contributes not only to the improvements in the overall performance, but also to the college persistence. The number of peer interaction partners in a high enrollment biology classes, termed the *degree of centrality* was positively associated with academic performance measured by GPA (Buchenroth-Martin, DiMartino, & Martin, 2017). Importantly, peer support, which resulted from establishing collaborative networks early in a student's college career, is an important factor contributing to the successful graduation of underrepresented minority STEM students (Lancaster & Xu,

2017). This study was aimed at exploring the effect of various collaborative learning approaches such as permanent or ad-hoc student groups organized for the purpose of short problem-solving sessions and low-stakes formative assessments (group quizzes), and group homework assignments on student engagement, satisfaction, academic performance, lecture attendance and retention. The effectiveness of the techniques was assessed using student surveys to inform and adjust the instruction. The data on student retention, attendance and success-failure rate was also gathered and analyzed. A decrease in the number of students who dropped or stopped attending the class and/or an increase in the passing rate was observed in the test group after the intervention aimed at increase in student interaction was implemented. The overall student satisfaction with the class fluctuated from semester to semester, and reflected conflicting feelings of students toward different types of group assignments. The findings indicate that community college student population respond better to more deliberate choice of instructional methods and faculty adapting instruction based on student feedback.

P906/B26

An Intensive Cellular Engineering Summer Research Course Promoting Interdisciplinary Learning.

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We report on the Cellular Engineering Summer Research Course offered by the Center for Cellular Construction and hosted at San Francisco State University. This course was designed to provide students an inclusive and intensive research experience on cellular engineering and cellular decision-making. Course goals emphasized development of skills across biology, engineering, and computational disciplines to prepare students to enter into modern scientific research programs. This inaugural course, “Summer of Cells 2019,” included 21 undergraduate, master’s and doctoral students who participated in three research projects during the 2-week course studying various aspects of cellular engineering, leading to discoveries in each area: *(1) Optogenetic control of cell migration*: Students found that immune cells use the rate of polarity factor production to make decisions about when to form protrusions and how frequently to change direction. *(2) Decision-making in Physarum*: Students studied how the morphology of the slime mold *Physarum* and its cytoplasmic flows allow this giant single-celled organism to solve complex optimization problems. *(3) Microfluidic manipulation of Stentor*: Students designed new microfluidic devices to cut single *Stentor* cells into multiple pieces, and they developed staining procedures to visualize *Stentor* structure. Retrospective pre- and post-surveys indicate that students showed growth in self-reported understanding of cellular engineering principles and cross-disciplinary techniques including: cell biology and genetics; optical microscopy; microfluidic design and fabrication; computational analysis; and scientific communication. Student co-authors: J. Aleman[2,7], G. Alvarez-Azanedo[2,7], J. Amatoru[2,7], J. Baker[2,8], A. Barrera-Velasquez[2,7], M. B. de Jesus[2,7], A. Chemel[2,7], G. Hale[2,9], H. Hernandez[2,10], W. Huang[2,7], K. Keeter[2,7], C. Kong[2,7], S. Namiranian[2,7], A. Nissenbaum[2,7], B. Oluoch[2,7], K. Padilla[2,7], A. Pereira[2,7], T. Quach[2,7], D. Ruiz[2,7], S. Sun[2,7], C. Weeks-Young[2,7] [8]UC Berkeley, Berkeley, CA, [9]St. Olaf College, Northfield, MN, [10]City College of San Francisco, San Francisco CA

P907/B27

Participation in Peer-led Team Learning (PLTL) in an Introductory Cell and Molecular Biology Course Correlates with Enhanced Outcomes for Both Students and Peer Leaders.

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Peer-led team learning (PLTL) is an active learning technique in which students work through complex topical problems in groups facilitated by a more experienced, trained student leader. In this study, we implemented optional, ungraded, out-of-class PLTL sessions in an introductory cell and molecular biology course and assessed outcomes for both students and peer leaders. The PLTL sessions were held nearly weekly for 60-75 minutes and paired groups of 6 - 8 students with one peer leader. The problems presented were related to topics being covered in the regular classroom periods at that time and generally included some interpretation of data from the primary literature, but were not intended to introduce new course content. Students who participated in more than 50% of PLTL sessions scored significantly higher on exams and had lower rates of D and F grades and withdrawals from the course than those who attended half or fewer of the PLTLs. In a post-course assessment, approximately 75% of students gave the peer-led problem sessions “good” or “excellent” ratings and reported that the PLTLs were effective at teaching problem-solving skills, facilitating the learning of course content, and integrating material from across the course. Peer leaders reported perceived gains by PLTL participants in understanding course material, ability to work through complex problems, and understanding data presented in the primary literature. Peer leaders also self-reported gains in their own understanding of course material, ability to work through biology problems, and interest and ability as a teacher. We are continuing to collect and analyze data in an effort to identify factors that enhance or detract from student participation and learning gains associated with PLTLs in this course.

P908/B28

Molecular Case Studies: at the Interface of Biology and Chemistry.

M. Lenahan; Raritan Valley Community College, Branchburg, NJ, NJ.

Title: Molecular Case Studies: at the Interface of Biology and Chemistry Abstract: “Structure and Function”, a biology education core concept, underscores the importance of structural units in defining the functions of living things. At a molecular level, structures of biological macromolecules (e.g., proteins and nucleic acids) define the molecular basis of their cellular functions. Experimentally determined structures of biological macromolecules and various visualization tools are freely available online. However, introducing students to molecular structure visualization, engaging them in structural analysis, and helping them bridge the gap between biology and chemistry to explain structure-function relationships, can be challenging. Case studies have often been used for active learning – to introduce new concepts/skills or to motivate student learning. Molecular case studies can provide contexts for teaching and learning structure-function relationships at the interface of biology and chemistry. A group of seven undergraduate educators from around the nation have formed a network, called Molecular CaseNet. They are collaborating to develop molecular cases for a variety of different courses and at different levels of rigor. These molecular cases provide a platform for integrating interdisciplinary knowledge from biology, biochemistry, chemistry, genetics, and cell biology. In addition, they engage students in “scientific practices” - e.g., introduce them to scientific literature, public biological databases, and bioinformatics tools and resources, many of which are currently underutilized in biology education. Molecular case studies cover a variety of topics. For example, “Nicholas' Story” is focused on

understanding changes in sickle cell hemoglobin leading to severe pain, its treatment using hydroxyurea, and gene therapy trials; while "A Case of Severe Insulin Resistance" explores defects in the insulin signaling pathway resulting in Type II diabetes. The ready to use cases can save undergraduate educators a lot of preparation time. Modular and flexible design of the cases enable students with varied levels of expertise and disciplinary training to explore molecular structure-function relationships, and apply the scientific process to address real-world problems. Over time, by using these case studies the next generation of STEM workers can be trained to creatively apply interdisciplinary approaches in problem-solving.

P909/B29

Perceived Efficacy of an Interactive Quizzing Tool for Learning Cell Biology, Histology and Pathology of George Washington University School by Medicine and Health Sciences (GWU-SMHS) Medical Students.

A. R. Munday, P. S. Latham, R. A. Jurjus; George Washington School of Medicine and Health Sciences, Washington, DC.

A move toward blended teaching in medical school curricula often results in decreased in-class time for disciplines such as Cell Biology, Histology and Pathology, as it has at GWU-SMHS. This study proposes to balance loss of in-class time with a self-study quizzing tool to increase medical student learning and comprehension of these disciplines, which are foundational in preclinical organ system studies. Since many studies have shown that quizzing increases learning, we developed an online, interactive, quizzing tool that allows students to test their ability to recognize visual features and to recall information. This study reports on the perceptions of medical students concerning the effectiveness of this tool as measured by survey. Images for the quizzing tool were derived from the MicroAnatomy and Pathology Atlas (MAPA) currently used by students for studies of histology and pathology (http://microanatomyatlas.com). The on-line quizzing tool was designed to display representative images without annotations or descriptions until activated by the student, allowing students to quiz themselves on the findings. The quizzing tool was provided to students in the class of 2022 near the beginning of each system block and used by them at their discretion. Students were invited to complete a survey asking if and how they used the quiz tool, and feedback about what they liked and did not like about the tool. A Likert scale (1-5) was used to rate the usefulness of the tool for their learning. The survey was given upon completion of the pulmonary organ block and again on completion of their first year. The quiz tool survey was first completed by 29.8% of students (56/188) after the pulmonary organ system block in 2019. The quizzing tool was rated by 60.7% of students at 5 (most useful) on the Likert scale, 30.4% rated it at 4, 7.1% at 3, 1.8% at 2, and 0% at 1. In answering the question 'Did using the quizzes make you more comfortable about answering histology questions on your exam?', 96.4% of respondents answered yes (54/56). After one year, the survey was repeated with a 17% student response rate (32/188), at this time 62.1% of the respondents rated the tool's usefulness at 5, 34.5% rated it at 4, and 3.4% rated it at 3. In answering 'Did using the quizzes make you more comfortable about answering histology questions on your exam?' 92.9% responded yes (26/28). These results indicate that the majority of students who completed the survey perceived that the quizzing tool had a positive impact on learning. The findings suggest that quizzing can enhance learning in cell biology, histology and histopathology, as it has been shown to do in other fields of learning. Future studies will use the quizzing tool to investigate not only perceived learning by students, but also the impact of using the tool on exam performance.

P910/B30

College of Arts and Sciences Faculty and Student Perceptions of Secondary Teaching as a Profession.

K. Baker, K. Stickney, D. Sachs; University of Indianapolis, Indianapolis, IN.

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the United States is continuing to face a critical shortage of qualified teachers, particularly in STEM (science, technology, engineering, and mathematics) disciplines. While there are many reasons for this shortage, one of the main factors for the emerging teacher shortage is a decline in enrollments in teacher preparation programs. Since many undergraduate students rely on faculty for career advice, the perceptions of faculty regarding secondary teaching as a career may play a role in either encouraging or discouraging a student's pursuit of teaching as a career. The purpose of this study was to investigate College of Arts and Sciences faculty and students' perceptions of secondary (grades 7-12) teaching as a profession. A survey was used to assess what factors would encourage or discourage STEM undergraduate students (N=271) from choosing a career in secondary teaching. Faculty (N=61) perceptions of secondary teaching were also assessed with the intent of extrapolating how those perceptions might impact recruiting for secondary teacher education programs. The results of this study support the importance of providing undergraduate STEM students and faculty advisors with accurate information about teacher salaries and the positive aspects of secondary teaching in order to encourage students to consider teaching as a career option.

New Techniques Using Fluoresence

P911/B32

Metadata and Performance Tracking for Fluorescent Microscopes I - Metadata.

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Microscopy images need to be accompanied by a description of the sample, its preparation and experimental layout as well as technical parameters under which images were taken. The term "metadata" is used to refer to such accompanying information, but the exact meaning of "metadata" frequently varies with context. A major challenge with metadata for technical parameters is the large variability of what is recorded by different microscopes. Metadata can be as simple as the pixel size or as complex as the results of an entire internal instrument calibration routine - and everything in between. To enable full quantitative analysis - to extract the maximal information content of images - and to make images from different microscopes comparable, we propose 1) an OME based, extended metadata model to capture complete hardware and settings used for image acquisition, 2) an extension of metadata to contain optical calibration- and performance documentation and 3) a tier system for metadata requirements that scales the amount of metadata to be reported with the complexity of the

imaging data. Here we present the proposed metadata model and tier system [1] and a tool [2] to collect metadata in a tier dependent manner through a GUI that enables non-experts to be thorough and complete in their metadata documentation of hardware and setting used. This work is accompanied by a second contribution on optical calibration and a third contribution on performance calibration. [1] https://github.com/WU-BIMAC/MicroscopyMetadata4DNGuidelines [2] https://github.com/WU-BIMAC/4DNMicroscopyMetadataTool

P912/B33

Metadata and Performance Tracking for Fluorescent Microscopes II - Optics.

D. Grunwald, M. Hammer, F. Farzam, M. Huisman; UMASS Medical School, Worcester, MA.

Microscopy images need to be accompanied by a description of the sample, its preparation and experimental layout as well as technical parameters under which images were taken. The term “metadata” is used to refer to such accompanying information, but the exact meaning of “metadata” frequently varies with context. A major challenge with metadata for technical parameters is the large variability of what is recorded by different microscopes. Metadata can be as simple as the pixel size or as complex as the results of an entire internal instrument calibration routine - and everything in between. To enable full quantitative analysis - to extract the maximal information content of images - and to make images from different microscopes comparable, we propose 1) an OME based, extended metadata model to capture complete hardware and settings used for image acquisition, 2) an extension of metadata to contain optical calibration- and performance documentation and 3) a tier system for metadata requirements that scales the amount of metadata to be reported with the complexity of the imaging data. Here we present cooperative data on the use of diffraction limited multicolor beads [1] and a number of imaging and resolution standards for optical calibration of a microscope [2-6]. This work is accompanied by a second contribution on a metadata model and a third contribution on performance calibration. [1] Theer, Mongis and Knop, “PSFj: know your fluorescence microscope”, Nature Methods (2014), 11:981; doi.org/10.1038/nmeth.3102 [2] https://github.com/cmongis/psfj/ [3]GellerRS4.2, http://www.gellermicro.com/mag_standards/MRS-4.2_190114.pdf [4] Argolight, http://argolight.com/products/argo-sim/ [5] Siemensstar, https://www.edmundoptics.com/p/high-resolution-microscopy-star-target/38664/ [6]PSFcheck, https://doi.org/10.1364/OE.26.021887, psfcheck.com

P913/B34

Metadata and Performance Tracking for Fluorescent Microscopes III - Metamax.

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Microscopy images need to be accompanied by a description of the sample, its preparation and experimental layout as well as technical parameters under which images were taken. The term “metadata” is used to refer to such accompanying information, but the exact meaning of “metadata” frequently varies with context. A major challenge with metadata for technical parameters is the large variability of what is recorded by different microscopes. Metadata can be as simple as the pixel size or as complex as the results of an entire internal instrument calibration routine - and everything in between. To enable full quantitative analysis - to extract the maximal information content of images - and to make

images from different microscopes comparable, we propose 1) an OME based, extended metadata model to capture complete hardware and settings used for image acquisition, 2) an extension of metadata to contain optical calibration- and performance documentation and 3) a tier system for metadata requirements that scales the amount of metadata to be reported with the complexity of the imaging data. However, there are certain crucial pieces of information that simply are not captured in even the most rigorous and precise routines for record-keeping and calibration, as they simply cannot be measured without the aid of (often costly, cumbersome and complicated) external devices. Here, we present an inexpensive, easy-to-use calibration device that, among other things, allows the user to measure excitation power and perform basic detector calibration routines. In doing so, the "MetaMax" tool provides crucial meta-data to evaluate potential photo-toxicity and allows current and future model-based data processing tools to get as much quantitative information as possible out of the images. MetaMax also provides an elegant, possibly automated way to track microscope performance over time. This work is accompanied by a second contribution on a metadata model and a third optical performance calibration. [1] Smith et al., "Probability-Based Particle Detection That Enables Threshold-Free and Robust in Vivo Single-Molecule Tracking", MBoC (2015), 22:4057-62; doi:10.1091/mbc.E15-06-0448. [2] Smith et al., "An automated Bayesian pipeline for rapid analysis of single-molecule binding data", Nature Comm. (2019), 10:272; doi.org/10.1038/s41467-018-08045-5

P914/B35

Combining Molecular Beacon and CRISPR Technologies for Sensitive Live-Cell Genomic Imaging.

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The ability to monitor the behavior of single genomic loci in living cells can offer tremendous opportunities for deciphering the molecular basis driving cellular physiology and pathogenesis. Toward this goal, clustered regularly interspersed short palindromic repeats (CRISPR)-based imaging systems have been developed, with tagging of either the nuclease-deactivated mutant of the CRISPR-associated protein 9 (dCas9) or the CRISPR single-guide RNA (sgRNA) with fluorescent protein (FP) molecules currently the major strategies for labeling. Recently, we have demonstrated the feasibility of tagging the sgRNA with molecular beacons (MB), a class of organic dye-based, fluorogenic oligonucleotide probes that have been used extensively for imaging endogenous RNA transcripts in living cells. We showed that, the resulting system, termed CRISPR/MB, enables accurate quantification of genomic loci, measurement of chromosomal dynamics, and dual-color genomic imaging. We should emphasize that, compared with conventional approaches that use FP molecules, CRISPR/MB may offer several advantages including brightness, photostability and reduced background (due to quenching when not bound to target). We are currently optimizing CRISPR/MB for ultrasensitive live-cell genomic imaging.

P915/B36

Development and Application of Designer RNA-binding Protein for Live-cell RNA Imaging and Manipulation of Authentic RNAs in Living Cells.

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RNAs do not only serve as the blueprint for the protein assembly, but also play wide variety of essential functions in cells. Furthermore, expression levels of mRNAs can be indicators of cell differentiation and signal transduction. Thus, visualization and manipulation of RNAs in living cells would be beneficial for

both basic and applied sciences. To this end, aptamer sequences such as MS2 stem loop or Broccoli, or synthetized fluorescent oligos such as molecular beacons were developed, however, modification of target RNA sequences and the lack of the method for efficient probe introduction have sometimes been critical problems. That's why fully genetically-encodable probes, which can label authentic, unmodified RNAs were expected to be developed.Here, we report the development of designable RNA-binding protein (dRBP), which is programmable to bind to the RNA of interest. We first established an ELISA-like in vitro assay system using our bright bioluminescent protein, Nano-lantern (Takai et al., *PNAS* 2015), and it was shown that the dRBPs have high affinity (1-10 nM) specifically to the target RNAs. To examine the avidity of our dRBPs to bind to target RNAs in living cells, we designed dRBPs for authentic, unmodified RNAs such as beta-actin mRNA. Immunoprecipitation of the probe followed by quantitative PCR analysis demonstrated that the target, authentic beta-actin mRNA was specifically recognized in vivo. We also showed that our dRBPs were applicable to the visualization of the dynamics of the authentic RNAs including beta-actin mRNA or long non-coding RNA Neat1_2 in living cells. Furthermore, manipulation of the localization of the beta-actin mRNA using the dRBP fused to constitutively active kinesin resulted in the neurite-like elongation of cellular processes. These data collectively suggest that our new probe for RNA would serve as a powerful tool for the imaging and manipulation of unmodified, authentic RNAs in living cells or organisms.

P916/B37

Imaging Endogenous Messenger RNA Expression Dynamics with an RNA Counterpart of a GFP.

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Single-cell analysis of the transcriptional dynamics gives us plenty of information about how a single cell contributes to the construction and the maintenance of the intricate biological systems. Fluorescent or luminescent protein-based reporters or RNA tagging systems with specific stem-loops that recruit fluorescent fusion proteins are commonly used strategies, but they indicate the transcriptional activity only indirectly. To circumvent this problem, we have recently developed an RNA counterpart of a GFP, Romanesco, which enables the direct and quantitative visualization of mRNA in living mammalian cells. Romanesco-based fluorescent reporter system has been applied to study cell-to-cell variance in transcriptional dynamics, mRNA incorporation into stress granules and RNA translocation during gene expression. However, because the copy number of the mRNA is usually much lower than that of the protein, visualizing mRNA at endogenous levels has been unsuccessful so far. Here we report new methods that enable the monitoring of the endogenous mRNA expression dynamics at single-cell resolution by greatly enhancing the detection sensitivity in Romanesco imaging. Our new methods realize the enhancement of the detection sensitivity by selectively eliminating the background signals derived from cellular autofluorescence and the fluorescence of the residual fluorophore not bound to Romanesco RNA. These methods rely on two photochemical properties of Romanesco: photoswitching and long fluorescence life time. In epifluorescence microscopy, Romanesco-derived fluorescence undergoes multiple rounds of on-off photoswitching whereas background fluorescence does not. Therefore by subtracting off-state images from on-state images background fluorescence is selectively eliminated. In laser-scanning microscopy, time-gated fluorescence lifetime imaging enables selective extraction of the Romanesco-derived fluorescence because Romanesco possesses an order of magnitude longer fluorescence lifetime compared to background fluorescence. By applying these methods to Romanesco imaging, more than 40-fold increase in signal-to-background ratio has been

achieved. We will discuss the details of these methods as well as their applications to the imaging of the endogenous mRNA in immune cells.

P917/B38

A FRET-Based Probe for High Throughput DNA Intercalator Drug Discovery and in Vivo Imaging.

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Molecules that bind DNA by intercalating its bases remain among the most potent cancer therapies and antimicrobials due to their interference with DNA-processing proteins. To accelerate the discovery of novel intercalating drugs, we designed a Fluorescence Resonance Energy Transfer (FRET)-based probe that reports on DNA intercalation, allowing rapid and sensitive screening of chemical libraries in a high-throughput format. We demonstrate that the method correctly identifies known DNA intercalators in approved drug libraries, and discover previously unreported intercalating molecules. When introduced in cells, the probe rapidly distributes in the nucleus allowing direct imaging of the dynamics of drug entry and its interaction with DNA in its native environment. The combined ability of the single probe to identify intercalators in vitro and follow their function in vivo can play a momentous role in accelerating the discovery of novel DNA-intercalating drugs or repurposing approved ones.

P918/B39

Fluorogen Activating Proteins as a Powerful New Imaging Tool for Quantitative Protein Trafficking Studies in Yeast.

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Recent advantages of genetically encoded fluorescent probes have led to the development of fluorogen activating proteins (FAPs). This technology has two components: a non-fluorescent single chain antibody (SCA) that can be fused to a protein of interest and fluorogens, which are non-fluorescent when free in solution. When the SCA and fluorogen bind, there is a 20,000-fold fluorescent increase relative to unbound dye. This level of fluorescence is comparable to GFP and other standard fluorescent proteins. However, the FAP-technology has two major advantages; (1) using either a membrane-permeant or impermeant fluorogen dye we are able to selectively label intracellular proteins from proteins at the permeable membrane and (2) since the fluorogen does not fluoresce when it is not bound by SCA, we are able to eliminate possible background fluorescence. Although developed in yeast, this technology had surprisingly not been used to detect localization in this model system until our recent work looking at the residence of membrane proteins at the cell surface. In order to make this technology more readily available to the yeast community, we have first optimized the SCA sequence for expression in yeast, then created a series of FAP tagging constructs for making FAP-tagged fusions to your favorite protein of interest, and finally have made a suite of FAP-tagged markers to be used as co-localization markers for the cell biology and yeast research communities. These tools will allow scientists to quantitatively analyze protein dynamics, minimize the effects of background, and selectively illuminate the surface or intracellular population of a tagged protein.

P919/B40

Live-cell an alysis of ER and Golgi Enzyme Activities in Gaucher Disease.

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TITLE: Live-Cell an alysis of ER and Golgi Enzyme Activities in Gaucher Disease INSTITUTION: Sonas BioPharma, 1850 Millrace Drive, Eugene, OR 97403 the Endoplasmic Reticulum (ER) and Golgi Apparatus are dynamic organelles that contain metabolic and regulatory enzymes involved in the ordered synthesis and processing of nearly all proteins, lipids, and carbohydrates in the cell. Functional deficiencies or mutations in these enzymes are indicative of a number of disease states, particularly those that affect the nervous system including the Lysosomal Storage Diseases and Parkinson's Disease. Methods to analyze Golgi and ER enzyme function within living cells have been hampered by the inability to monitor activity within individual organelles. We developed new, organelle-targeted, live-cell fluorogenic substrates for Golgi, ER and lysosomal enzymes that can be used for in *situ* analysis of changes in their metabolic activity and intracellular trafficking for the Lysosomal Storage Diseases. Staining with these new probes was shown to co-localize with known markers of lysosomal, ER and Golgi morphology. We have now utilized these targeted fluorogenic substrates to perform a pilot screen with a focused library of compounds known to modulate protein transport, act as pharmacological chaperones or otherwise alter enzyme levels or trafficking in the ER, Golgi and lysosome. Using both fibroblasts and B-lymphoblast cells derived from Gaucher I patients alongside control cells from healthy individuals we were able to compare the GCase activity in the ER and the lysosome in response to drug application by staining with the targeted substrates. We identified a new, potential hit from this screen, compound SB1282. Application of 10 uM SB1282 to Gaucher I cells for 72 hours resulted in a 92% decrease in GCase β-glucosidase activity in the ER with a corresponding greater than 50% increase in enzyme activity in the lysosome as compared to untreated controls, indicating improved intracellular trafficking of the enzyme from the ER to the lysosome. We are currently performing SAR studies to develop analogs of SB1282 with improved efficacy, employing intracellular enzyme activity analyses with our targeted fluorogenic substrates, as well as by monitoring ER stress (UPR) utilizing antibody co-staining (ASK1 CHOP, GRP78, or XBP1: Abcam) and measuring total lipid burden changes by HPLC analysis. Our screening methodology is being similarly employed with existing larger libraries to elucidate additional potential hits. This work was funded in part by NIH-NIGMS grant 5R44GM108137-03.

P920/B41

CatCh-ER: an Endoplasmic Reticulum Membrane-Localized Optogenetic Channel for the Manipulation of ER Calcium.

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The endoplasmic reticulum (ER) is the site of protein synthesis, lipid metabolism, cellular signaling, and calcium storage for the cell. Its role as a calcium store greatly facilitates and is indeed necessary for its other functions. Dysregulation of ER calcium homeostasis has been implicated in numerous human diseases, ranging from neurodegenerative to metabolic to infectious. In order to better understand the role of ER calcium in disease pathogenesis, I present here the development and characterization of CatCh-ER, a modified optogenetic channel with increased calcium permeability compared to ChR2 and localized to the ER membrane. CatCh-ER provides increased spatiotemporal control of modulating ER calcium, allowing for more precise study of the role of ER calcium dysregulation in disease.

P921/B42

Brief Calcium Influx through Membrane Channels Transiently Stabilizes Pip2and Recruits Actin in the Inner Plasma Membrane Leaflet of Intact Cells.

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Calcium entering the cell through ion channels acts as fast diffusion signal molecule. However, local calcium levels near the membrane can reach several hundred micromoles, sufficiently high to act electrostatically. The inner membrane leaflet contains various negatively charged lipids, such as phosphatidylinositol 4,5- bisphosphates (PIP2) and Phosphatidylinositol (3,4,5)-trisphosphate (PIP3). These modulate protein function and recruit specific proteins to the plasma membrane. In model membrane systems it has been shown that Calcium ions induce clusters of PIP2. Hence, we have investigated whether Calcium influx modulates the lateral PIP3 and PIP3 distribution in intact cells. This could cause very quick rearrangements during cell signaling and modulate the bilayer to cytoskeleton linkage, and hence be important for mechanosensitive channels, such as Pieoz1. However, it makes imaging the membrane faithfully very challenging. We employed fluorescent correlation spectroscopy (FCS) measuring diffusion on multiple length scales simultaneously, to show that the transient calcium in flux leads to a transient clustering of PIP2 and PIP3 on the inner membrane leaflet. To study the formation of PIP2 clusters in the plasma membrane we use GFP-PHPLCdeltaand Halo-PHPLCdelta labelled with the JaneliaFluor647; as marker for PIP3 PH-AKT-GFP; and for cholesterol-stabilized nano-domains Lck-mGFP. We find that opening TRPV1 or Piezo1 channels leads to a transient rise in calcium as imaged using GCaMP5G, transiently stabilizes PIP2 and PIP3 with PIP3 following a slower kinetics. It also increases the interaction between the Lck-mGFP and cholesterol domains. Using an ionophore to clamp the calcium level to a fixed value, we determine the threshold for these effects. We find that vinculin in rest cells stabilizes PIP2 clusters. In vinculin knockdown cells the calcium induced clustering and redispersion are accelerated. These results suggest a concentration dependence of calcium-induced PIP2 clusters and cholesterol- stabilized nano-domains in the PM at calcium levels, which may be reached in intact cells locally by opening of ion channels.

P922/B43

Comparative an alysis of Taxol-derived Fluorescent Probes to Assess Microtubule Network Organization in Drosophila Egg Chambers.

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Drosophila oogenesis is an excellent in vivo model for investigating cytoskeletal dynamics because of the rapid cytoskeletal remodeling that occurs at the end of stage 10. While there are several genetic tools to fluorescently label the actin cytoskeleton with minimal artifacts, there are few robust tools for detecting microtubules in live complex tissues. The recent development of membrane permeable microtubule binding taxol-based fluorescent probes is significant technical progress; however, the effectiveness of these probes and the potential stabilizing effects of the taxol derivative have not been well characterized in vivo. Here, we compared ViaFluor (Biodium), Tubulin Tracker (ThermoScientific), and SiR-Tubulin (Spirochrome), three commercially available taxol-derived microtubule probes coupled to far red emitting dyes to determine their efficacy and potential artifacts. We assessed whether the three probes labeled microtubules with differences in the speed of penetration, brightness, and signal to noise ratio. Like taxol, however, all of the probes disrupted the actin cytoskeleton in a concentration

dependent manner. The manufacturer's instructions for SiR-tubulin recommend using the efflux pump inhibitor, verapamil. We found that verapamil increased both the brightness for SiR-Tubulin, and the severity of the actin artifacts. In the Drosophila ovary, Tubulin Tracker was the brightest of the three dyes, and we demonstrated that washing out the dye after a 30 minute incubation maintained sufficient signal brightness over the 90 minute imaging period and significantly reduced the actin artifacts. Together these data suggest that careful optimization including a taxol control is essential to ensure that signal is optimized while minimizing artifacts due to unintentional microtubule stabilization.

P923/B44

Engineering Biologically-relevant Boundaries: Optochemical Patterning of a Synthetic Cell Boundary to Probe Spindle Positioning.

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In all multicellular organisms, proper development and adult tissue renewal rely on precise cell divisions. Cell division defects are associated with developmental disorders, neurological defects, and cancer, and they are linked to mispositioning of the mitotic spindle during anaphase. Mutations have been identified in a suite of proteins, including tumor suppressors Adenomatous polyposis coli (APC) and p53, that are thought to function in spindle positioning. This suggests that disruption of spindle positioning can lead to cell division defects and subsequent disease. However, a major challenge has been characterizing the functional roles of these proteins and how their mutation contributes to disease pathology. Studies in living cells are confounded by the presence of tens of thousands of distinct macromolecules and the complex nature of their interactions. An approach that overcomes these hurdles is to characterize minimal components in emulsion systems, which match the geometry of cells. I encapsulated recombinant optochemical proteins and microtubules inside water-in-oil emulsions. The optochemical system consists of FRB and FKBP fusion proteins and a photocaged dRap. Upon 405 nm laser illumination, dRap is uncaged and free rapamycin promotes dimerization of FKBP and FRB, thereby translating an optical input to a biochemical output. After 1 second of illumination, GFP-FRB protein is quickly relocalized to the boundary to generate an anisotropic boundary. I light-inducibly recruited microtubule adapter proteins (i.e., LGN) and GFP-FRB fusion proteins in a similar manner to examine their effects on spindle microtubule position in a minimal context. An additional advance is that we can incorporate cell-free systems that can carry out aspects of the cell cycle in *vitro*, including spindle assembly. By leveraging emulsion, cell-free extract, and optochemical approaches, we can pattern a synthetic boundary and build spindle interactions from the bottom-up to characterize the dynamics and spatial patterning of molecular factors required for proper cell division. This platform will provide new insights into the molecular mechanisms underlying spindle positioning, and ultimately generate paradigms to explain how defects arise in cell division and contribute to disease.

P924/B45

EGFP Is a Lifetime Highlighter for Fluorescence Lifetime Imaging (FLIM).

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Enhanced green fluorescence protein (eGFP), a successor of the wild type GFP from *Aequorea victoria*, is a popular and trusted fluorescent tag extensively used in biological and biomedical research. Over the

years researches gathered extended collections of cell lines bearing specific eGFP-tagged proteins. Surprisingly, some photochemical properties of eGFP have remained unexplored. We report on a lifetime-based photoconversion of eGFP especially usable in fluorescence lifetime imaging. Our results document that eGFP can be permanently photoconverted to a short-fluorescence-lifetime form (PC-eGFP) either by intense blue irradiation or by a two-photon process. PC-eGFP exhibits the same spectral emission compared to the unconverted form. Fluorescence of PC-eGFP is pH-independent and the photoconversion efficiency depends on the solvent viscosity and presence of external redox agents. Besides potential FLIM artifacts caused by an unintended photoconversion, the controlled process turns eGFP to an excellent tool for kinetic FLIM applications. This is demonstrated by tracking of a nucleophosmin in live cells during its cytoplasm-nuclear relocalization induced by external drugs. Since the photoconversion occurs in the lifetime domain, PC-eGFP is easily distinguishable from the unconverted tag by time-resolved detection. All other spectral detection channels stay free for multicolor labeling. Reported properties line up eGFP with other photoconvertible fluorescent proteins with special advantage in FLIM where lifetime-photoconvertible labels are scarce. (This work was supported by the Czech Science Foundation, grant 19-04099S).

P925/B46

***In Vivo* Multiplexed Bioimaging of Intrinsic and Extrinsic Biomarkers.**

H. J. Chiang, E. S. Koo, S. Cai, W. Shi, P. Wang, L. A. Trinh, S. E. Fraser, **F. Cutrale**; University of Southern California, Los an geles, CA.

There is a complex relationship between signaling pathways, extracellular microenvironment, different tissues and metabolic requirements of the cell. These intertwined factors contribute to an orchestrated communication across scales, from cells to tissues, which plays an important role during development and in a wide range of diseases. The palette of fluorescent proteins has been expanded, covering a wide range of colors. However, the time course of dynamics remains unclear due to a technological imaging gap. To study these multiplexed processes, imaging techniques need to simultaneously satisfy multiple opposing needs: capability to separate spectrally overlapping fluorophores, sufficient sensitivity to image at single-cell resolution without perturbing the process under study, and fast enough to capture the 3-dimensional volume within which the cell is interacting. Spectral imaging has the potential to fulfill this need by offering an increased informational domain that can capture the intertwined multi-scale complex biological interactions. In this work, we report our novel methods for analyzing fluorescent data in the multi-spectral domain. Using novel implementation of Machine Learning, denoising, unmixing and phasors, we enable rapid lossless denoising of spectral data and enable parallel observation of multiple intrinsic and extrinsic biomarkers.

P926/B47

DISC: Quantitative Light Sheet Fluorescence Microscopy with Minimal Shadowing.

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Live-cell fluorescence microscopy has enabled biologists to observe subcellular dynamics at high spatial resolution. However, traditional fluorescence microscopy modes introduce unnecessary photodamage to the fluorescent sample. A recent revolution in fluorescence microscopy, Light Sheet Fluorescence Microscopy (LSFM), drastically decreases the rate of photodamage to live organisms by illuminating the sample from the side. Unfortunately, this illumination scheme introduces shadowing artifacts in the final

image, complicating any quantitative analysis of fluorescence intensity. To address the shadowing problem inherent in LSFM, we propose DISC (Diametric Illumination for Sheet Consistency) microscopy. DISC microscopy uses a parabolic mirror to illuminate fluorescent samples with a converging light sheet from every angle, rather than a single light sheet from one angle. The 360º illumination reduces shadowing to its theoretical minimum, making DISC microscopy ideal for maintaining a consistent excitation intensity while still spatially confining the excitation light to a single plane. Additionally, DISC microscopy is compatible with coverslip-mounted samples to maximize fluorescence detection efficiency with high-numerical aperture objectives. Thus, DISC microscopy nearly eliminates shadowing artifacts in LSFM while reducing photodamage in live-cell fluorescence microscopy.

P927/B48

Mapping Protein Counts in Live Cells.

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Calibrating Fluorescence Microscopes allows estimates of the number of proteins in each pixel of a 3D image stack from living human induced Pluripotent Stem Cells (hiPSC). We applied this method to several cell lines from the Allen Cell Collection expressing monomeric enhanced Green Fluorescence Protein (mEGFP) tagged proteins that highlight major structures[1]. To determine protein counts from fluorescence intensities, we imaged mEGFP solutions at six concentrations (from 22-1787nM) using settings optimized for cell imaging on the Zeiss spinning disk (Zeiss Cell Observer SD) and super-resolution microscopes (Zeiss LSM 880 Airyscan Fast). We confirmed the mEGFP concentrations using Fluorescence Correlation Spectroscopy (FCS) and then fitted linear regression to intensity data from solution images and FCS determined concentrations. We applied this calibration curve to fluorescence images of cells to estimate protein count per pixel. To ascertain that fluorescence properties of mEGFP are identical in cells and calibration solutions, we used number & brightness (N&B) analysis. The brightness parameter of this fluctuation-based method is proportional to quantum efficiency of the dye, given fixed imaging parameters. Since most of the Allen Cell Collection have one allele tagged with mEGFP, we measured the relative amount of mEGFP labeled protein and endogenous, unlabeled protein using immunoblotting for the protein and mEGFP. The relative level of tagged to non-tagged protein is close to 50% for most cell lines; however, there are outliers (e.g., NPM1 (22%), LMB1(33%))[2]. We corrected these calibration curves using the ratio of tagged to non-tagged protein. To validate the method, we used cell lines expressing cytosolic mEGFP, estimating the concentration of mEGFP with point-FCS and quantitative immunoblots from cell extracts. In general, the data are within a factor of 2, e.g., 68±35nM for FCS measurements in the cytosol, 117±18nM from the immunoblots from cell extracts, and 73±24nM by our method. In summary, we have developed an image-based, sub-cellular protein count method that can be easily applied to proteins through three orders of expression levels and multiple microscope modalities. FCS measurements were not greatly limited by cell movement, bleaching, and phototoxicity, thus allowing robust fitting and higher concentrations. However, for microscopes without FCS capability, the measurements can be calibrated using solutions of known concentration. [1)] https://www.allencell.org/cell-catalog.html [2)] Roberts, B., et al. (2017). Mol Biol Cell 28(21): 2854-2874.

P928/B49

New Non-curing Mounting Media Couples Improved Axial Resolution with Cross-spectrum Photobleach Protection Enabling High Quality Deep Tissue Imaging.

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Fluorescence microscopy offers unparalleled insight into the inner workings of specimen tissue by illuminating sub-cellular structures. The ability to visualize and interrogate specimen tissue is complicated by factors including photostability and tissue thickness. These factors are more pronounced when imaging thicker tissue, >30 microns, which is often of greater interest than thinner tissue samples. The longer signal acquisition times required for thicker tissue place increased demand on the signal-generating fluorphores leading to a greater potential for photobleaching. Fluorophore photobleaching occurs after repeated excitation cycles and results in the loss of signal. To mitigate photobleaching, reagents such as free-radical scavengers and antioxidants are included in the mounting media to minimize fluorophore degradation. Image distortion is caused by poor optical matching between the lens objective, glass cover slip, and the sample itself. The refractive index (RI) is a characterization of how materials in the signal path interacts with light, and any mismatch in this pathway limits the axial resolution and focal depth of the imaging process. Cover glass and immersion oil objectives (RI=1.52) minimize refraction of light traveling to and from the sample. Most commonly used mounting media have a RI near 1.45 resulting in a mismatch. In thicker biological samples it is vital to match the refractive indexes of your materials to acquire a high-quality image. Images acquired with mismatched materials are blurry, distorted, and lack the desired focal depth. Presented here is a new mounting media formulation with an RI=1.52 developed to provide both protection against photo-degradation and RI-matching in a non-curing format. To demonstrate resolution improvement with RI-matching, point spread functions of sub-resolution microspheres were measured to quantitatively compare samples mounted with the new mounting media and current alternatives. Images of 100 micron thick tissues were mounted with the new formulation and compared to non-curing mounting media with RIs=1.45. The ability to look deeper into samples and cross-spectrum photobleach protection enables acquisition of high-resolution images of thick tissue specimens. These experiments seek to educate and empower microscopy users with additional considerations when choosing antifade mounting media.

New Techniques in Cell Biology 2: Centers, Education, Outreach

P930/B51

Best Practices at the National Center for Cryo-EM Access and Training.

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Advances in cryo-electron microscopy (cryo-EM) imaging technology and data processing have resulted in the recent growth of single particle structures, which extend to near-atomic resolution. To broaden biomedical scientists' access to cryo-EM the NIH Common Fund's Transformative High Resolution Cryo-Electron Microscopy program has created three national service centers to provide access to the technology and the development of training curricula to build a skilled workforce. The mission of the NCCAT (National Center for Cryo-EM Access and Training) service center is twofold: to provide nationwide access to advanced cryo-EM technical capabilities, and to assist users in the development of cryo-EM skills needed for independent research. NCCAT provides access to state-of-the-art equipment required to solve structures to the highest possible resolution using cryo-EM methods. By implementing

the most current best practices users are able to optimize different experimental parameters on-the-fly, thereby allowing reconstructions to be determined from images collected in a single day. Our objective is to enable biomedical researchers from all fields to make use of these cryo-EM techniques and methodologies in their research programs.

P929/B50

The Allen 3d Live Cell Imaging Pipeline: Automation, Standardization and Quality Control.

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The Allen Institute for Cell Science combines 3D live cell imaging of cellular spatial and temporal organization with genomics to identify and define cell states and to unlock how cells transition among various normal and pathological states. We do this using a collection of genome-edited human induced pluripotent stem cell (hiPSC) lines expressing green fluorescent protein tagged to proteins identifying specific cellular organelles and structures (**The Allen Cell Collection at Allencell.org**). Using these cells, we visualize the 3D organization and dynamics of cellular structures under diverse and defined conditions, creating a holistic picture of the changes in cellular organization. To collect and process these data we developed a highly standardized, quality controlled and automated 3D live cell imaging pipeline. Here, we describe the standardization and automation of our imaging workflow, which includes 5 distinct steps: 1) Imaging sample preparation, 2) sample & region of interest selection, 3) high resolution image acquisition, 4) post-acquisition processing, and 5) 3D image segmentation. Each step is optimized and automated to increase reproducibility and minimize error introduced by repetitive manual tasks. We first developed a robotic human iPSC culture platform to generate uniform and reproducible imaging plates. Each plate is ranked based on confluency and cell morphology using an algorithm that segments and classifies cell colonies. Coordinates from the center and edge of each colonies are selected and 3D images automatically captured from this position list using Zeiss spinning disk confocal microscopes operated with Zen Blue software and open-application development scripts. The data flows through an image processing pipeline to generate individually segmented 3D cells and is stored in a data management system built to manage the high volume of data produced. To maintain quality, we implemented rigorous quality control over cell morphology, microscope optics, image data and segmentations. The laser power at the objective, field homogeneity and point-spread functions are measured daily to ensure optimal performance. Image and cell qualities are curated using criteria like cell and colony morphology, absence of debris, whole cell integrity and signal-to-noise ratio. Algorithms are now being developed to further automate these final processes.

P931/B52

Supporting New Technology Development to Accelerate the Pace of Cancer Research: the Innovative Molecular an alysis Technologies (IMAT) Program.

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The National Cancer Institute (NCI) established the Innovative Molecular an alysis Technologies (IMAT) Program in 1998 to support the development of technologies to accelerate the pace of discovery and address challenges facing basic and clinical cancer researchers. The program has become a technology development pipeline that supports the genesis, technical maturation, and dissemination of novel next-generation technologies through an approach of balanced but targeted innovation. Among the strengths of the IMAT Program is the diversity of disciplines from which potentially transformative technologies

are solicited, including technologies to advance research in cancer etiology, epidemiology, cellular mechanics, biophysics, biospecimen science, and the analysis of cancer development and progression. Technology development is supported at several levels, from early conceptual stages to technique validation to commercialization. The IMAT Program empowers translational research by taking risks on early stage technologies, many of which have matured into methods widely applicable across research disciplines. Some of these include elegant molecular biology tools to detect the presence of proteins, biomarkers, and miRNAs with high sensitivity as well as novel biosensors and imaging tools to observe cellular features in real time. The IMAT Program supports the implementation of these novel technologies through additional funding opportunities for researchers with active NCI grant awards to integrate these tools into their ongoing basic and clinical research projects. The IMAT Program's support for the development of research tools and platforms accelerates basic cancer research and translates these discoveries into tomorrow's patient care.

P932/B53

3d Image-based Data Generation Pipeline: Effect of Drug Perturbations on the Allen Cell Collection.

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The Allen Institute for Cell Science combines 3D live cell imaging of cellular spatial and temporal organization with genomics to identify and define cell states and to unlock how cells transition among various normal and pathological states. We do this using a collection of genome-edited human induced pluripotent stem cell (hiPSC) lines expressing green fluorescent protein tagged to proteins identifying specific cellular organelles and structures (**Allen Cell Collection at allencell.org**). In addition to normal and pathological organization, the Institute is also interested in developing a holistic 3D image database of cellular responses to a variety of perturbations including well-characterized drugs commonly used to perturb specific cellular processes, structures, or pathways. This database is being used to study and elucidate inter-dependency among cellular structures, train models to identify perturbed cell states, and identify likely pathways influenced by mutations. Our cell culture and imaging pipeline workflow has been fully automated using a Hamilton star robotic platform and a spinning disk confocal microscopy platform. The workflow also includes semi-automated quality control processes. Phenotypes observed in the images are first qualitatively described, and subsequently, 3D image segmentation and feature extraction are performed and used for quantitative analysis. Here we present a growing 3D image-based dataset matrix to include multiple drug perturbations collected at 3 different time points (2, 4 and 24 hrs post-perturbation) over different cell lines of the Allen Cell Collection. Through the parallel use of qualitative and quantitative methods, we plan to create a 3D image-based map of cell structure co-variation. Ultimately, the perturbation signature and structure co-variation will allow us to propose candidate cell structure inter-dependency and predict the directionality of these relationships.

P933/B54

Identification of Biosamples Using NIDDK Information Network (dkNET) Resource Reports: an Integrated Resource Network Platform That Helps Improve Rigor and Reproducibility.

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Awareness of problems with scientific reproducibility has risen in recent years. A survey of 1576 researchers showed that more than 70% of researchers failed to reproduce experiments done by other

scientists [1]. To improve reproducibility, the National Institutes of Health announced new guidelines for rigor and reproducibility in grant applications in 2016. The identification of research resources is an essential step to enhance reproducibility. For example, without a unique identification system for biosamples, one cannot cross-reference the source of biosamples across experiments between different projects or organizations. Research Resource Identifiers (RRIDs), unique identifiers for research resources, help improve identification of research resources in the biomedical literature [2]. As of August 2019, there are more than 600 Journals that have RRID citations, including endocrinology society journals. Utilizing RRIDs, dkNET(NIDDK Information Network; dknet.org)[3] created Resource Reports to help researchers identify key resources such as digital tools, antibodies, cell lines, and organisms, and track resource use and performance. Using text mining and data aggregation technologies, the reports provide detailed information about research resources along with citation metrics from the biomedical literature, community feedback, alerts when the resources have problems, and information about who is using the resource. In this project, we expanded the resource types to include biosamples. To identify the human pancreatic islets used by the Integrated Islet Distribution Program (IIDP) researchers, the Resource Identification Initiative worked with the BioSample databases in the EU and the US and the IIDP team to develop a registration pipeline. Biosamples are registered with the NCBI Biosample database to obtain BioSample SAM* identifiers which are used as RRIDs. Researchers can now easily find useful resource information on pancreas islets and track their usage via dkNET Resource Reports. RRIDs improve the identification of biosamples and researchers can cite the RRIDs when publishing papers. Researchers can not only pool or re-analyze the data across experiments and studies, but can aggregate data from different biosamples from the same donor. Two diabetes relevant journals, Diabetologia and Diabetes, now require researchers to cite biosamples using unique identifiers. Researchers are encouraged to contact distributor programs to register their biosamples. Based on RRIDs, we can also integrate more data to provide updated information. References: (1) Baker M. Nature. 2016; 533: 452-4. (2) Bandrowski AE et al., Neuron. 2016; 90(3):434-6. (3) Whetzel PL et al., PLoS One. 2015; 10(9):e0136206.Source of Support: NIH NIDDK Grant U24DK097771

P934/B55

The NIH Common Fund's Extracellular RNA (exRNA) Communication Program.

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The NIH Common Fund's Extracellular RNA (exRNA) Communication Program was developed to address critical issues in exRNA research. The overarching objective is to generate a multi-component community resource for sharing fundamental scientific discoveries, protocols, and innovative tools and technologies. Key components of the first stage of the exRNA Communication Program include (a) generating a reference catalogue of exRNAs present in human body fluids, (b) defining the fundamental principles of exRNA biogenesis, distribution, uptake, and function (c) identifying exRNA biomarkers of diseases, (d) demonstrating clinical utility of exRNAs as therapies, and (e) developing a resource to provide the scientific community access to exRNA data, standardized protocols, and other tools and technologies. The second stage of the exRNA Communication Program will address specific technological challenges including (a) the development of molecular tools, methods and technologies for single-vesicle characterization and (b) scalable methods for separation and analysis of exRNA carrier subclasses. Ongoing outreach efforts will provide the greater scientific community with these exRNA resources, novel tools and technologies. Recent progress to be presented include showcasing new community-wide tools for researchers. ExRNA data sets are being generated and shared to be findable,

accessible, interoperable and reusable (FAIR). New exRNA tools and technologies to be presented include the Extracellular RNA Communication Consortium's Virtual Biorepository, the miRDaR web application for RNA isolation, the ExceRpt small RNA-seq analysis pipeline, and the exRNA Atlas. Author list: https://commonfund.nih.gov/Exrna/members Resources: https://exrna.org/; https://commonfund.nih.gov/exrna

P935/B56

Cryoem-101: a Media-rich Online Curriculum for Learning About the Cryo-em Workflow.

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Cryo-EM has emerged as a powerful tool for the high-resolution determination of macromolecular structures. We have created a media-rich online curriculum, called CryoEM 101, to aid the training efforts of newcomers to the field. Using videos, animations, and interactive simulations, CryoEM 101 provides self-paced learning material that introduces students to the protocols, expectations, limitations and troubleshooting of standard procedures of a typical cryo-EM project. The course is divided into five chapters: (1) sample purification, (2) cryo-EM grid preparation, (3) cryo-EM grid screening, (4) data collection, and (5) image processing and analysis. All material will be made freely available at http://cryoem101.org.

P936/B57

The NIH Common Fund Human Biomolecular Atlas Program (HuBMAP): Building a Framework for Mapping the Human Body.

D. C. Procaccini, R. S. Conroy; Common Fund, NIH, Bethesda, MD.

Normal 0 false false false EN-US X-NONE X-NONE /* Style Definitions */ table.MsoNormalTable {mso-style-name:"Table Normal"; mso-tstyle-rowband-size:0; mso-tstyle-colband-size:0; mso-style-noshow:yes; mso-style-priority:99; mso-style-parent:""; mso-padding-alt:0in 5.4pt 0in 5.4pt; mso-para-margin-top:0in; mso-para-margin-right:0in; mso-para-margin-bottom:8.0pt; mso-para-margin-left:0in; line-height:107%; mso-pagination:widow-orphan; font-size:11.0pt; font-family:"Calibri",sans-serif; mso-ascii-font-family:Calibri; mso-ascii-theme-font:minor-latin; mso-hansi-font-family:Calibri; mso-hansi-theme-font:minor-latin; mso-bidi-font-family:"Times New Roman"; mso-bidi-theme-font:minor-bidi;} Understanding how tissue organization influences a cell's molecular make-up, interactions, and history is vital to elucidating variation in organ function across lifespan and health-disease continuum. Even with cutting edge imaging and omics technologies, current knowledge of how tissues are organized is restricted to a small number of microscopic structures. Better insights into principles governing organizational and functional relationships will potentially provide a better understanding of inter-individual variability, lifespan changes, tissue engineering approaches, and the emergence of disease at the biomolecular level. However, high throughput integration of imaging and omics analysis to comprehensively profile biomolecular distribution and morphology in tissues is challenging. Further, placing this information on 3D tissue maps amenable to modelling and molecular perturbation has yet to be fully realized. HuBMAP, in conjunction with many single-cell consortia doing single-cell work, is working to catalyze the development of a comprehensive atlas of cellular organization in humantissues to meet this challenge.

Actin and Actin-Associated Proteins 2

P937/B59

Role of Cyclase-associated Protein in Actin Dynamics.

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The most prominent force-producing machinery in eukaryotic cells is the actin cytoskeleton. It powers diverse cellular processes through rapid polymerization of actin filaments at their barbed ends that is balanced by filament disassembly at pointed ends. While much is known about how actin filaments grow, we know substantially less about mechanisms of their disassembly. The best known actin disassembly factors are the cofilin family proteins, which increase cytoskeletal dynamics by severing actin filaments. However, the mechanism by which severed actin filaments are recycled back to monomeric form to fuel actin filament growth has remained enigmatic. Here we determined the molecular mechanism of actin filament pointed end depolymerization. We show that a ubiquitous actin regulator, cyclase-associated protein (CAP), binds actin filament pointed ends through its HFD domains, and works in synergy with cofilin to increase actin filament pointed end depolymerization by nearly 100-fold. By determining the crystal structure of HFD domain in complex with actin, combined with single filament imaging and atomistic molecular dynamics simulations, we revealed that CAP depolymerizes actin filaments by destabilizing the interface between the two terminal actin subunits at filament pointed end. Moreover, we show that the HFD domain can deliver the newly depolymerized actin monomer to the C-terminal half of CAP, which subsequently recharges the monomer with ATP. Our work establishes CAP as a molecular machine that accelerates actin monomer dissociation from filament pointed ends, and subsequently recycles actin monomers for new rounds of filament assembly. Together, these findings unveil that rapid actin dynamics relies on both actin filament severing and pointed end depolymerization, and explain why CAP is critical for dynamic actin-dependent processes in all organisms ranging from unicellular eukaryotes to multicellular plants and animals.

P938/B60

Metabolic Alterations in the Hearts of Cyclase Associated Protein 2 Mutant Mice.

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Cyclase associated proteins (CAPs) are actin binding proteins that regulate the cytoskeleton and associated signaling pathways such as SRF. The CAP2 isoform is expressed mainly in heart, skeletal muscle and brain. Loss of CAP2 causes dilated cardiomyopathy (DCM) and conduction delays, but the effects of CAP2 loss on metabolism has not been studied. We performed a metabolomics analysis to investigate the effects of CAP2 loss on energy metabolism in mouse hearts. Female mice (n=4/group, 1-year old) from CAP2 KO and age-matched control mice (n=4/group) were analyzed for acyl carnitines (fatty acids) and organic acids (lactate, pyruvate and TCA cycle intermediates) in heart tissue. CAP2 KO mice exhibited a strikingly different metabolic signature in their fatty acid and organic acids profile. Fatty acid profiling showed a significant increase ($p<0.05$) in long-chain fatty acids in CAP2 KO mice compared with controls and a trend towards increased levels of short chain fatty acids between the groups. Lactate and pyruvate levels were also increased ($p<0.05$), but the lactate/pyruvate ratio was decreased

in CAP2 KO mice in comparison to control mice. In addition, all the intermediates of the TCA cycle were significantly increased (except for citrate, being increased) in CAP2 KO mice. RNAseq data also showed significant decrease in genes (*Ech1, AcadM, Acaa2, Slc27a1, Acsl6,* etc.) associated with fatty acid oxidation, uptake, transport and lipid modifications. Decreased citrate levels associated with increased pyruvate and lactate levels suggest the flux of glycolysis towards lactate formation instead of the TCA cycle in CAP2 KO mice. However, increased levels of other intermediates of TCA cycle and accumulation of fatty acids might be due to anaplerotic reactions to refuel TCA cycle and decreased fatty acid oxidation respectively as supported by RNA seq data. Therefore, CAP2 mutation leads to strikingly different metabolic signature characterized by a surge towards anaerobic glycolysis and decreased fatty acid oxidation. This metabolic signature could be an additional factor aggravating the pathology in DCM patients.

P939/B61

Small Cofilin Clusters Sever Actin Filaments and Grow Preferentially at the Pointed End Independent of Filament Tension.

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Actin filaments comprise force generating networks that facilitate various cellular functions such as migration and division. Filament network turnover is accelerated by members of the ADF/cofilin family of regulatory proteins that sever filaments and increase the concentration of filaments ends available for growth and disassembly. Cofilin severs filaments at junctions, or “boundaries”, between bare and cofilin-decorated segments. Pulling forces have been used to visualize cofilin binding and investigate force sensitivity, but have yielded conflicting behaviors. We used microfluidics to apply tension on actin filaments and directly visualized cooperative cofilin binding, cluster formation and filament severing with TIRF microscopy. We observed preferential cofilin cluster growth toward filament pointed ends. Small clusters (less than three cofilin) displayed severing activity. Filament bending, flow-induced tension, and attachment conditions did not substantially affect cofilin association kinetics. This work addresses the discrepancies in the field on cofilin force sensitivity, cluster growth, and severing.

P940/B62

Diversification in the ADF-H Domain Family of Actin Regulatory Proteins.

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Cofilin and Abp1 are ubiquitous, abundant actin-binding proteins that both use Actin Depolymerizing Factor Homology (ADF-H) domains to bind the sides of actin filaments, yet have highly distinct regulatory effects on F-actin. Cofilin severs and depolymerizes filaments, whereas Abp1 instead promotes Arp2/3 complex-dependent actin nucleation and stabilizes branch junctions. This raises important questions, including: Do these ADF-H proteins compete for binding to F-actin? Can they co-exist on filament sides at the same time? Do they interfere with each other’s functions? to begin to address these questions, we performed in *vitro* multi-wavelength TIRF imaging using labeled yeast Cofilin (Cof1) and Abp1. We found that Cof1 and Abp1 each bind to filaments with similar affinities (Kd ~ 0.5 μM), but highly distinct kinetics. Cofilin binds and dissociates slowly, whereas Abp1 binds and dissociates rapidly (sub-second time scale). Even though these proteins compete for overlapping binding sites on F-actin, when mixed at their normal cellular ratio and concentrations, they do not interfere with each other functionally. Abp1 initially coats filaments in a non-cooperative manner, then Cofilin slowly

binds, in a cooperative manner, to form spots and induce severing. Thus, the unique kinetic properties of these two ADF-H proteins allow them to sequentially perform their functions in each other's presence. Our observations predict that mutating Cofilin in *vivo* to reduce its dwell time on filaments will result in defects caused by Abp1 competition and interference. Indeed, we found that *cof1-22*, which weakens F-actin binding, causes cellular defects in actin organization and function that are suppressed by *abp1Δ*. These results shed new light on the diversification of ADF-H family proteins, and how complex systems in cells have evolved to employ multiple proteins with a related binding module, tuning their properties so that they perform distinct functions while working in harmony with each other.

P941/B63

Actin-binding Proteins Modulate the Activity of Actin-specific Adp-ribosylating Toxins.

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Bacterial pathogens have developed multiple strategies to disrupt the actin cytoskeleton, and thereby compromise numerous cellular processes assisted by actin. Several actin-targeting ADP-ribosylating toxins (ARTs) from various pathogens covalently modify one of two residues on actin - R177 or T148. ADP-ribosylation at R177 converts actin monomers (G-actin) into non-polymerizable species, leading to the cytoskeleton disruption. In contrast, modification of T148 supports actin polymerization and stabilizes filamentous actin (F-actin), resulting in abnormal actin dynamics and accumulation of F-actin aggregates. ADP-ribosylation by ARTs has classically been studied using G-actin as a substrate, however, under physiological conditions, G-actin is typically partnered with thymosin β4 (TMSB4) or profilin (PFN1). In this study, our goal was to evaluate the activity of ARTs with physiologically relevant G-actin substrates. In this study, ADP-ribosylation was monitored by following either changes in fluorescence of etheno-NAD^+ or via a shift in actin mobility on the native-PAGE upon its modification by ARTs. We found that among three R177-ARTs from different bacterial species, PFN1-actin complex is a preferred substrate for *Salmonella enterica* ART SpvB; whereas *Aeromonas hydrophila* VgrG1 and *Clostridium perfringens* Iota toxins showed no substantial preference toward PFN1-actin or TMSB4-actin. Deletion of the poly-proline rich motifs from SpvB and VgrG1 toxins reduced the PFN1's effect on the efficiency of the enzymes, in agreement with the hypothesis that poly-proline regions contribute to the binding of these ARTs to the actin-PFN1 complex. R177 modification caused no effect on the affinity of TMSB4 or PFN1 to G-actin. Given the fast exchange of actin between TMSB4- and PFN1-bound complexes, the substrate preference should not selectively affect PFN1-dependent or -independent actin polymerization pathways. Surprisingly, accumulation of R177-ADP-ribosylated-actin in cells inhibited the mDia1-controlled actin polymerization substantially faster than the leading-edge retrograde flow of actin controlled by the Arp2/3 complex and myosin contractility. For *Photorhabdus luminescens* T148-ART TccC3, F-actin and G-actin were modified with similar rates, but the presence of TMSB4 or PFN1 prevented modification of G-actin, suggesting that F-actin is the physiological substrate of TccC3. We confirmed previous reports that T148-ADP-ribosylated actin is resistant to severing by another essential actin binding protein cofilin and demonstrated a stoichiometric character of this resistivity. The multifaceted roles of actin binding proteins in the toxicity mechanisms of ARTs and other actin-specific toxins remain to be elucidated.

P942/B64

Dissecting Profilin Mediated Actin-microtubule Interactions by Storm.

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Super-resolution microscopy methods are powerful tools that break the diffraction barriers of conventional light microscopy, allowing for higher spatial resolution and visualization of cellular structures in greater detail (typically expanding resolution from microns to nanometers). We used Stochastic Optical Reconstruction Microscopy (STORM) and ThunderSTORM software to visualize actin, microtubules, and cytoskeletal regulatory proteins, specifically Profilin-1 and Profilin-2. Our most optimal imaging conditions permitted ~10-fold increase in resolution of actin filaments from ~200 nm obtained using conventional TIRF and/or confocal microscopy to ~20 nm with STORM. Here we show optimized conditions to visualize these proteins in mouse Neuroblastoma 2A (N2A) cells. While each Profilin isoform associated with both actin and microtubules, Profilin-1 was more strongly colocalized with the actin-rich regions of the cell and Profilin-2 more prevalent on the sides of microtubules. We are currently investigating how these localizations change with specific mutations in Profilin-1 and Profilin-2 that disrupt actin and microtubule-binding.

P943/B65

Profilin-1 Controls Actin Network Assembly, Organization, and Homeostasis.

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An increasingly important question for the field is how actin binding proteins coordinately regulate the cytoskeleton. Yet the rules that determine how complex networks form from multiple assembly factors remain poorly understood. Profilin-1 (PFN1) buffers the actin monomer pool and promotes polymerization through interactions with polymerases, allowing it to bias assembly of specific types of actin networks. In this study, we sought to understand how PFN1 controls actin assembly, organization, and homeostasis in the elaborate environment of mammalian cells. In PFN1 knock-out (KO) cells, the ability to polymerize actin through Arp2/3 and formins is partially lost, while assembly by Mena/VASP is completely inhibited. We also found PFN1 is necessary to maintain monomer/filament homeostasis, even when Arp2/3 or formins are inhibited. PFN1 KO cells also have depleted lamellipodia actin and a loss of Arp2/3 localization to the leading edge. By reintroducing discrete amounts of PFN1 protein, we identified a concentration-dependent relationship of PFN1 to lamellipodia architecture. Lower profilin concentrations stimulate the formation of filipodia and linear filament arrays and allow Arp2/3 to re-localize to the leading edge, while higher concentrations stimulate dendritic network assembly. These findings demonstrate a nuanced, coordinate action of PFN1 with other actin binding proteins, and suggest that filaments generated through PFN1 create the majority of Arp2/3 binding sites. This study provides new insight into how PFN1 controls the assembly of complex actin networks.

P944/B66

Gelsolin-Mediated Actin Filament Severing in Crowded Environments.

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Actin is an essential cytoskeletal protein that plays a key role in several cellular functions such as phagocytosis and cell motility with the help of actin binding proteins (ABPs). Gelsolin is a calcium

regulated ABP that severs and caps actin filaments. These two proteins control actin assembly and disassembly, dynamic processes that are required for cell survival. The majority of in *vitro* studies of gelsolin and actin have been performed in dilute buffer conditions that do not properly model the intracellular environment which is crowded with high concentrations of macromolecules. These macromolecules induce depletion forces and excluded volume effects. We hypothesize that gelsolin and actin filaments present in crowded environments will lead to greater severing activity due to the excluded volume effects. To test this hypothesis, co-sedimentation assays were performed in order to determine the effect of macromolecular crowding on the binding affinity of gelsolin to actin. We have directly visualized actin filament severing by gelsolin in solution including macromolecular crowders utilizing total internal reflection fluorescence (TIRF) microscopy. Steady-state average filament lengths as well as filament length distributions were analyzed to determine the effect crowding has on gelsolin-mediated filament severing. Real-time filament severing assays allowed us to estimate the filament disassembly rates in the presence of macromolecular crowding. Taken together, this study demonstrates that macromolecular crowding modulates gelsolin-mediated actin filament severing activities and offers insights into the function of both actin and gelsolin inside the cell.

P945/B67

Regulation of Inf2-mediated Actin Polymerization through Site-specific Lysine Acetylation of Actin Itself.

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INF2 is a formin protein that accelerates actin polymerization and also severs actin filaments. A common regulatory mechanism for formins is autoinhibition, through interaction between the N-terminal diaphanous inhibitory domain (DID) and C-terminal diaphanous autoregulatory domain (DAD). We recently showed that INF2 utilizes a variant of this mechanism which we termed ‘facilitated autoinhibition’, whereby a complex consisting of cyclase-associated protein (CAP) bound to lysine-acetylated actin (KAc-actin) is required for INF2 inhibition, in a manner still requiring DID. We also showed that de-acetylation of actin in the CAP/KAc-actin complex activates INF2. Here, we use lysine-to-glutamine mutations as acetyl-mimetics to map the relevant lysines on actin for INF2 regulation, focusing on three positions: K50, K61 and K328. Biochemically, purified K50Q-, K61Q and K328Q β-actin polymerize with similar equilibrium and kinetic properties to WT β-actin or rabbit skeletal muscle (RSK) actin, both in the absence and presence of INF2. When complexed with CAP2, however, K50Q- and K61Q- inhibit RSK-actin polymerization by full-length INF2, but not by INF2 lacking DID. When expressed at low levels in U2OS cells, K50Q- and K61Q-actin inhibit INF2-mediated actin polymerization. Direct binding studies show that the CAP WH2 domain binds INF2-DID with sub-micromolar affinity but has weak affinity for actin, while INF2-DAD binds CAP/K50Q-actin 5-fold better than CAP/WT-actin, suggesting an inhibition model whereby CAP/KAc-actin serves as a bridge between INF2 DID and DAD. In U2OS cells, INF2 is 70 and 8-fold less abundant than CAP1 and CAP2, respectively, suggesting that there is sufficient CAP for full INF2 inhibition.

P946/B68

Cellular Mechanisms Controlling *S. Cerevisiae* Formin Displacement from Barbed Ends.

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Formins are highly processive actin machines, and in the absence of other cellular factors stay attached to growing barbed ends for minutes, assembling filaments that are 50-100 times longer than those found in vivo. This raises an important question: how do cells tune the duration of formin activity to control filament length? Recently, we showed that mammalian capping protein forms 'decision complexes' with formins at barbed ends, catalyzing formin displacement to limit growth. Here we asked whether related mechanisms are used in *S. cerevisiae* to control the activities of its two formins (Bni1 and Bnr1). Bni1 and Bnr1 have distinct biochemical properties, localize to different cellular sites, and assemble distinct sets of actin cables, suggesting that these two formins may be differentially regulated in vivo. We show that *bni1Δ* (but not *bnr1Δ*) displays strong synthetic growth defects with loss of Cap1/2, suggesting that Cap1/2 plays an important role in regulating Bnr1 in vivo. Microfluidics-assisted TIRF microscopy further revealed that Bnr1 can be readily displaced from barbed ends by Cap1/2, or by the Bnr1-binding partner Bud14, and that *bud14Δ* and *cap2Δ* mutants display strong synthetic defects in cell growth and actin organization. Taken together, our results suggest that Bnr1 can be displaced from barbed ends by two distinct and genetically complementary mechanisms, and that formin displacement may be more critical for controlling cable formation by Bnr1 than Bni1. Currently, we are using single molecule imaging to determine whether Cap1/2 and/or Bud14 join Bnr1 at barbed ends in decision complexes, and to define the similarities and differences in their displacement mechanisms.

P947/B69

Fhod-1 and Muscle Cell Size in *Caenorhabditis Elegans*.

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Formins are cytoskeletal proteins best known for their ability to nucleate actin and to remodel the actin cytoskeleton within cells. A mammalian formin, FHOD3, is required for cardiac muscle cell maturation in mice. In neonatal rat cardiomyocytes, activated FHOD3 leads to hypertrophy, whereas knockdown of FHOD3 leads to disrupted sarcomere formation. In humans, FHOD3 variants have been linked to an increased incidence of hypertrophic cardiomyopathy. However, how FHOD3 contributes to muscle cell size remains unknown. Using a simple genetic model, *Caenorhabditis elegans,* we are investigating how FHOD-1, a homolog of FHOD3, contributes to muscle cell size. Our lab has shown that in *C. elegans,* loss of FHOD-1 is not lethal, unlike the loss of FHOD3 in mice. Worms lacking FHOD-1 develop striated muscles that are thinner beginning in mid-larval stage and never recover. We have shown that loss of FHOD-1 leads to the reduction of muscle-specific myosin heavy chain 3 (MYO-3) protein expression. Previous work has shown that formins regulate muscle gene expression indirectly through enhancing transcription in mammalian systems. To determine if FHOD-1 promotes MYO-3 mRNA or muscle gene expression, we preformed qRT-PCR and mRNA sequencing. However, our qRT-PCR and mRNA sequencing results showed that MYO-3 mRNA and muscle-specific genes were not downregulated in worms lacking FHOD-1. Thus, removing transcription as a means of FHOD-1 regulating muscle cell size. To determine if MYO-3 protein is being degraded, we pharmacologically inhibited two protein degradation pathways: calpains and the proteasome. Preliminarily, we have seen calpains have no effect on MYO-3. However, the proteasome appears to degrade MYO-3 protein. It is our hope that by

determining the role of FHOD-1 in the regulation of muscle protein expression, we might better understand how FHOD3 contributes to hypertrophic cardiomyopathy.

P948/B70

FHOD-1 Is the Only Formin That Contributes Directly to the Development of Body Wall Muscles in *Caenorhabditis Elegans*.

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The striated body wall muscles (BWMs) of *Caenorhabditis elegans* are a simple model system with well-characterized sarcomeres that have many vertebrate protein homologs. Previously, we observed that the deletion mutants for two formin genes, *fhod-1* and *cyk-1,* had thinner BWMs compared to wild type. We wanted to more fully characterize how FHOD-1 and CYK-1 contribute to BWM development. However, CYK-1 is essential for cytokinesis during embryonic development, so homozygous *cyk-1(Δ)* progeny are derived from a heterozygous parent from which they receive a maternal contribution of CYK-1. We suspected that the presence of maternal CYK-1 in homozygous *cyk-1(Δ)* renders it partially functional and contributes to the development of BWMs. To test the complete loss of CYK-1 and avoid the issue of maternal CYK-1, we used a fast-acting temperature sensitive *cyk-1(ts)* mutant. Surprisingly, post-embryonic loss of CYK-1 had a minimal effect on larval BWM growth whereas loss of FHOD-1 has a more major effect. Additionally, only loss of FHOD-1 caused dispersed dense bodies which are Z-line analogs. During embryonic sarcomerogenesis, we observed that acute loss of CYK-1 did not cause any gross BWM defects, but the loss of FHOD-1 caused the F-actin striations to appear frayed. To test for cell autonomous roles in BWM development, we created mosaic formin expression strains by re-expressing either full-length FHOD-1 or CYK-1 in *fhod-1(Δ) and cyk-1(Δ)* mutants, respectively. We observed that individual BWM cell size and dense body morphology in *fhod-1(Δ)* mutants were rescued by re-expressing FHOD-1 in BWM cells. However, there was no similar rescue in *cyk-1(Δ)* mutants. This suggests that FHOD-1 promotes muscle cell growth cell-autonomously, whereas CYK-1 functions in a non cell-autonomous manner likely during embryonic development and its effect on BWMs could be indirect.

P949/B71

Mdia1/3 Facilitate Lat Phosphorylation by Zap70 Via the Spatiotemporal Control of F-actin Polymerization at the Immune Synapse.

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The mechanism by which the cytosolic protein Zap70 physically interacts with and phosphorylates its substrate, the transmembrane protein LAT, uponT cell receptor (TCR) stimulation remains largely obscure. In this study, we found that the pharmacological inhibition of formins, a major class of actin nucleators, suppressed LAT phosphorylation by Zap70, despite TCR stimulation-dependent phosphorylation of Zap70 remaining intact. High resolution imaging and 3D image reconstruction revealed that localization of phosphorylated Zap70 to the immune synapse (IS) and subsequent LAT phosphorylation are critically dependent on formin-mediated actin polymerization. Using knockout mice, we identify mDia1 and mDia3, which are highly expressed in T cells and which localize to the IS

upon TCR activation, as the critical formins mediating this process. Our findings therefore describe previously unsuspected roles for mDia1 and mDia3 in the spatiotemporal control of Zap70-dependent LAT phosphorylation at the IS through regulation of filamentous actin, and underscore their physiological importance in TCR signaling.

P950/B72

Effect of Arpc1b Deficiency on Neutrophil Directed Migration.

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Neutrophils are primary cells of the innate immune system that mediate host defense. Neutrophils exhibit rapid, directed migration in response to chemoattractants and tissue damage. We have shown that the Arp2/3 complex is critical for neutrophil directed migration in vivo. We have also found that Arp2/3 inhibition impairs neutrophil directed migration in microfluidic systems in vitro. Here, we characterize the role of the ArpC1b, a WD40 repeat containing protein and regulatory component in the Arp2/3 complex on neutrophil directed migration. Previous studies have described patients with inflammation and allergy induced by Arpc1b deficiency. We modeled this disease in neutrophil-like PLB-985 cells using CRISPR/Cas9 to deplete Arpc1b. We found that Arbc1b depletion severely impaired neutrophil directed migration in 2D, and moderately impaired migration in a 3D matrigel environment. Expression of Arpc1b-GFP in the deficient line rescued cell migration. Taken together, these findings suggest that Arpc1b is necessary for efficient directed migration of neutrophil-like cells.

P951/B73

Capping Protein Insulates Arp2/3-assembled Actin Patches from Formins.

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How actin structures of distinct identities and functions co-exist within the same environment is a critical self-organization question. Fission yeast cells have a simple actin cytoskeleton made of four structures: Arp2/3 assembles actin patches around endocytic pits; the formins For3, Cdc12 and Fus1 assemble actin cables, the cytokinetic ring during division, and the fusion focus during sexual reproduction, respectively. The focus concentrates the delivery of hydrolases by myosin V to digest the cell wall for cell fusion. We discovered that cells lacking capping protein, a heterodimer that blocks barbed-end dynamics and associates with actin patches, exhibit a delay in fusion. Consistent with capping protein-formin competition for barbed-end binding, Fus1, F-actin and the linear filament marker tropomyosin hyper-accumulate at the fusion focus in cells lacking capping protein. Capping protein deletion also rescues the fusion defect of a mutation in the Fus1 knob region. However, myosin V and exocytic cargoes are reduced at the fusion focus and diverted to ectopic foci, which underlies the fusion defect. Remarkably, the ectopic foci coincide with Arp2/3-assembled actin patches, which now contain low levels of Fus1. We further show that capping protein localization to actin patches is required to prevent the formation of ectopic foci and promote efficient cell fusion. During mitotic growth, actin patches lacking capping protein similarly display a dual identity, as they accumulate the formins For3 and Cdc12, normally absent from patches, and are co-decorated by the linear filament binding-protein tropomyosin and the patch marker fimbrin. Thus, capping protein serves to protect Arp2/3-nucleated structures from formin activity.

P952/B74

Structure-activity Relationships and Mechanism of the Formin Inhibitor Smifh2.

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The small molecule drug SMIFH2 was discovered in a biochemical screen for inhibitors of formin-stimulated actin polymerization (Rizvi, et al. 2009). While the original in *vitro* screen was carried out against the mouse formin mDia1, SMIFH2 was also found to be a cell permeable inhibitor that targeted both yeast and mammalian formins. Since then, SMIFH2 has been used widely to investigate formin function in diverse cellular contexts. However, the molecular mechanism of inhibition remains mysterious, and it is unknown whether the drug effects formin-mediated actin-microtubule crosstalk. In this study, we measured the affinity of SMIFH2 for purified C-terminal fragments from a range of human formins. SMIFH2 was found to have broad activity against human formins. To explore the relationship between chemical structure and biological activity, we generated analogs that vary in each ring of the molecule. Consistent with published data, a sulfur carbonyl on the central ring is essential for high-affinity binding. Our analog series also shows that the oxygen in the furan ring is essential and that halogenation but not methylation of the furan is tolerated. Neither INF2 or FMNL3 are able to bind to microtubules when bound to SMIFH2. Together our data support a model of SMIFH2 as a pan-formin inhibitor that blocks both actin and microtubule binding. Rizvi, S. A., Neidt,E.M., Cui, J., Feiger, Z., Skau, C.T., Gardel, M.L. Kozmin, S.A., Kovar D.R., Chemistry & Biology 2009, 16: 1158-1168.

P953/B75

Wiskott-Aldrich Syndrome Protein (wasp) Is Critical in Regulating Actin Reorganization Upon BCR Activation in Germinal Center B Cells.

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B-lymphocytes are responsible for generating antibody responses against pathogens. B-cell receptors (BCRs) sense antigen and initiate B-cell activation. Activated B-cells form germinal centers (GC), where B-cells with high-affinity BCRs were selected through their competitive ability to engage and capture antigen on antigen-presenting cells. Actin plays a critical role in BCR activation in naïve B-cells. Actin drives B-cell spreading and subsequent contract on antigen-presenting cells, enhancing and attenuating BCR signaling, respectively, by reorganizing receptors. How actin works during the selection of GC B cells (GCBs) remains unclear. The hyperactivity of GCBs in Wiskott-Aldrich syndrome protein (WASP)-deficient mice supports a distinct role for actin in GCBs. Using WT and WASP knockout (WKO) mice expressing Lifeact-GFP or GFP-non-muscle myosin II as models, this study examined actin's role in BCR activation in GCBs. We quantitatively analyzed the dynamics of actin reorganization, cell spreading, and BCR clustering in GCBs interacting with antigen on planar lipid bilayers (PLBs) using live-cell imaging by total internal reflection fluorescence and interference reflection microscopy. We found that WT GCBs rapidly accumulate F-actin at the contact zone upon binding the stimulatory PLB. After the accumulation reached a plateau, a central actomyosin ring structure formed. Concurrently, GCBs spread rapidly and symmetrically. Upon reaching a plateau, the contact area was maintained. The periphery area of the contact zone bound to the PLB much stably than the central area. As GCBs spread, BCRs rapidly accumulated and formed microclusters in the contact zone. Upon forming, BCRs microclusters did not grow or merge. Compared to WT GCBs, both the kinetics and the maximal levels of F-actin accumulation

in the contact zone of WKO GCBs were significantly reduced. There was also no central actomyosin ring structure. WKO GCBs took a longer time to take foothold to the PLB. Upon binding, WKO GCBs spread asymmetrically in a slower rate than WT GCBs and could not maintain the stably bound periphery and the active center in the contact area. Consequently, BCRs clustered at a slower rate and a reduced level in the contact zone of WKO GCBs, which reduced the kinetics and levels of signaling. Our results suggest that actin in GCBs is not only responsible for driving cell spreading and BCR clustering, but also for maintaining the sizes and organization of BCR microclusters for optimal signaling.

P954/B76

The Actin Regulatory Protein WASp Control Activation of Nucleic Acid Innate Sensors.

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Wiskott-Aldrich syndrome protein (WASp) is an actin nucleation promoting factors (NPFs) specifically expressed in cells on the hematopoietic lineage that controls multiple immune related functions. Mutations is WASp are linked to a rare immune deficiency, Wiskott-Aldrich syndrome (WAS), that manifests with frequent autoimmune manifestations and increased inflammatory responses to self-DNA in myeloid cells. At present the exact molecular link between actin regulation and excessive activation of inflammatory genes is not fully understood. Here we show that endosomal F-actin nucleation by Wiskott-Aldrich syndrome protein (WASp) is required for endosomal sorting and fusion with lysosomes in dendritic cells. Lack of WASp induces stalling and accumulation of ingested immunogenic cargo in maturation-defective organelles enhancing their immunogenicity. Moreover, defective endosomal maturation is accompanied by increased membrane fragility and leakage of endocytosed material to the cytosol, favoring activation of cytosolic innate sensors. These findings identify WASp as critical to set the threshold of innate sensors and unveil the mechanism underlying excessive inflammatory responses in WAS disease.

P955/B77

Elucidating Mechanisms Contributing to Actin Meshwork Formation.

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Temporally and spatially controlled actin filament assembly and disassembly is essential for a wide range of cellular processes. In *Drosophila* oogenesis, two actin nucleators, Spire and Capu (FMN-formin family member), are critical for meshwork formation. The mammalian homologs of Spire and Capu (FMN2) are required for a similar meshwork in mouse oocytes, along with the GTPase Rab11 and the actin motor protein MyosinV (MyoV). Recently, MyoV was shown to recruit Spire to Rab11-positive vesicles through a direct interaction in mouse. Not only do Spire, Rab11 and MyoV form a tripartite complex, this complex also recruits FMN2 and provides the first model of coordination between cytoskeleton assembly and transport. However, the ability of Spire or FMN2 to assemble actin filaments while part of this complex remains to be tested. To investigate the significance of Spire-MyoV binding in *Drosophila,* we first examined their binding in *vitro* and have identified key residues for their interaction. We report the effects of MyoV binding on Spire actin assembly in *vitro.* Together, these data form the basis for further studies in *Drosophila* oocytes to understand actin meshwork formation.

P956/B78

Inhibition of Arp2/3-mediated Actin Nucleation Alters Membrane Dynamics but Does Not Block Pigment Granule Aggregation Or Dispersion in Isolated Fish Retinal Pigment Epithelial (RPE) Cells.

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Actin filaments support organelle transport in numerous cell types by serving as tracks for multiple classes of myosin motors. In addition, non-muscle myosin II has been shown to power endosome, mitochondria, and actin patch movement in budding yeast indirectly, by moving actin cables to which organelles are attached. A similar mechanism has been proposed for aggregation of pigment granules through apical projections of fish retinal pigment epithelial (RPE) cells. Dissociated RPE cells cultured in vitro, having their apical projections attached to the substrate can be triggered to aggregate pigment granules towards the cell body through the application of cAMP. Aggregation of pigment granules requires an intact actin cytoskeleton and non-muscle myosin II. To further investigate the mechanism of pigment granule aggregation, and determine the role of actin nucleation in pigment granule motility, we treated isolated cells with the Arp2/3 complex inhibitor, CK-666. Time lapse videos revealed that in control cells, RPE apical projections are very dynamic, often exhibiting random protrusions of membrane and cytoplasm that extend laterally from RPE cell projections, then move in a retrograde direction, towards the cell center. Treatment with 100 uM CK-666 rapidly stopped formation and movement of these membrane protrusions. However, aggregation of pigment granules in RPE cells treated with CK-666 occurred as in control cells, even after a 30 minute pre-treatment with the drug. Similarly, CK-666 did not block dispersion of pigment granules out of the cell body. These results suggest that although membrane dynamics in RPE apical projections is affected CK-666, pigment granule motility persists, and is independent of actin filament nucleation.

P957/B79

Arp2/3 Nucleates Conventional and Divergent Actin Networks In*Chlamydomonas Reinhardtii*.

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Because conventional actin is critical for many cellular processes, it is generally highly conserved, but divergent actins do exist. Here, we investigate the interactions between Arp2/3, a branched actin nucleator, and actins with varying degrees of variance using the unicellular green alga *Chlamydomonas reinhardtii,* which expresses two actin genes: *IDA5*, a conventional actin that is 89% identical to rabbit skeletal muscle actin; and *NAP1,*a divergent actin with only about 65% sequence identity. Sequence alignments between IDA5 and NAP1 show that of the 30 actin residues that bind Arp2/3, over half are not conserved in NAP1. Based on these differences, we hypothesized that Arp2/3 would interact with IDA5 but not NAP1. To test this in *vivo*, we identified structures and phenotypes dependent on either IDA5 or NAP1 to use as readouts of interaction. We found IDA5 dependent puncta that may be analogous to actin patches or endocytic pits and flagellar assembly and maintenance were Arp2/3 dependent. This data supports the first half of our hypothesis that Arp2/3 can nucleate IDA5 filaments. Next, we tested whether Arp2/3 could interact with NAP1 by looking at NAP1-dependent structures and phenotypes. LatB-induced NAP1 perinuclear rings, NAP1-dependent flagellar assembly and maintenance under conditions free of IDA5 filaments, and NAP1-dependent restoration of gating proteins at the base of the flagella were inhibited by Arp2/3 perturbation. This data suggests that Arp2/3 can nucleate filaments of NAP1 disproving our hypothesis that Arp2/3 would not interact with NAP1. Overall, we

conclude that although sequence variance exists between the Arp2/3 binding regions of IDA5 and NAP1, chemical and genetic disruption of Arp2/3 affects organization and function of filaments made of either actin. This suggests potential interactions between IDA5 and NAP1 with Arp2/3 which we aim to biochemically characterize.

P958/B80

Photoreceptor Disc Membranes Are Formed through a Lamellipodium-like Mechanism.

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The light-sensitive outer segment of the vertebrate photoreceptor is a highly modified primary cilium filled with disc-shaped membranes which provide a vast surface for efficient photon capture. The formation of each disc is initiated by a ciliary membrane evagination driven by an unknown molecular mechanism reportedly requiring actin polymerization. Since a distinct F-actin network resides precisely at the site of disc morphogenesis, we employed a unique proteomic approach to identify components of this network potentially driving disc morphogenesis. Most importantly, the only identified actin nucleator was the Arp2/3 complex, which induces the polymerization of branched actin networks. Conditional knockout of Arp2/3 from rod photoreceptors results in the complete loss of the F-actin network specifically at the site of disc morphogenesis, and completely halts the initiation of new disc formation. These data establish a new model of photoreceptor disc morphogenesis in which Arp2/3 initiates disc formation in a “lamellipodium-like” mechanism.

P959/B81

Deep Learning Reveals the Link between Filament Architecture and Subunit Conformation in Bent Actin.

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Migrating cells can be “steered” by mechanical cues in their local microenvironments on rapid timescales through direct mechanical regulation of the branched actin networks powering motility. The binding probability of the ARP2/3 complex, which nucleates daughter filament branches from the side of pre-existing mother filaments, was previously shown to be biased by mother filament curvature, suggesting a mechanism to couple network density to filament bending by compressive load. We pursued cryo-EM structural analysis of bent filaments, hypothesizing it could reveal structural states of the actin subunit which could be recognized by binding partners at the molecular scale. We found that standard single-particle classification methods to probe structural heterogeneity poorly captured bent states of actin, which occur infrequently under standard sample preparation conditions and differ too subtly from straight actin to be separated by maximum-likelihood based approaches. We thus developed a deep-learning based approach to identify bent actin segments: F-actin was curved in silico and synthetic projection images were generated. A denoising autoencoder was trained to learn a continuous manifold of actin bending from these images. The encoding portion of the network was then used to help train two other neural networks: one network performed semantic segmentation on entire micrographs, and another network measured filament curvature on a per-particle basis. These trained networks were used to pick actin segments with curvatures measured above a fixed threshold, which were then used to reconstruct a bent F-actin state to near-atomic resolution. This structure shows substantial rearrangements of the actin subunits in the bent region which could be recognized by

binding partners including ARP2/3. This study also represents, to our knowledge, the first high-resolution glimpse at a high-energy, mechanically-regulated structural state, providing insight into how mechanical forces are transduced into protein conformational regulation in atomistic detail.

P960/B82
Insights into Actin Polymerization and Nucleation Using a Coarse-grained Model.
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We studied actin filament polymerization and nucleation with molecular dynamics simulations and a previously established coarse-grained model having each residue represented by a single interaction site located at the C_α atom. We approximate each actin protein as a fully or partially rigid unit to identify the equilibrium structural ensemble of interprotein complexes. Monomers in the F-actin configuration bound to both barbed and pointed ends of a short F-actin filament at the anticipated locations for polymerization. Binding at both ends occurred with similar affinity. Contacts between residues of the incoming subunit and the short filament were consistent with expectation from models based on crystallography, X-ray diffraction and cryo-electron microscopy. Binding at the barbed and pointed end also occurred at tilted conformations and the barbed end tilt range was dependent on the flexibility of the D-loop. Additional barbed end bound states were seen when the incoming subunit was in the G form. Consistent with an activation barrier for pointed end polymerization, G-actin did not bind at an F-actin pointed end. In all cases, binding at the barbed end also occurred in a configuration similar to the antiparallel (lower) dimer. Individual monomers bound each other in a short-pitch helix complex in addition to other configurations, with several of them apparently non-productive for polymerization. Simulations with multiple monomers in the F-actin form reproduce filamentation as well as transient aggregates at the barbed end. We discuss the implications on the kinetic pathway of actin filament nucleation and polymerization and possibilities for future improvements of the coarse-grained model.

Regulation of Actin Dynamics 2

P961/B83
Regulators of Arp2/3 Nucleation Are Combinatorially Deployed to Build Complex 3D Actin Structures in *Vivo*.
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Despite extensive studies on the actin regulators that direct microfilament dynamics, how these regulators are combinatorially employed to generate complex three-dimensional structures in an intact organism is an unsolved question. Here, we present an in-depth characterization of actin cap dynamics and their regulation in vivo in the early Drosophila syncytium. Actin caps go through rapid phases of initiation, expansion, elongation and fragmentation in each syncytial cycle. We examine the role of 7 different Arp 2/3 regulators and find they provide distinct but cooperative functions in building actin cap morphologies. Specifically, while DPod1 is the major regulator of actin intensities in the apical cap, Cortactin is required for the continued growth of the cap as well as the maintenance of cap size. Coronin, on the other hand, functions in both cap size and intensity, and, intriguingly, is required for Cortactin localization to the cap periphery. Surprisingly, knocking down the function of Formin or Arp2/3 networks leads to faster FRAP recoveries, suggesting that these networks are in deep competition for a limited G-actin pool. We also measure the relative efficiencies of Arp2/3 recruitment by each regulator

in the embryo. Finally, proper structuring of the actin cap is essential for genomic stability and nuclei anchorage, with a loss of actin/intensity/levels/size correlating with nuclear fallout. Our results suggest how the coordination of multiple actin regulators can orchestrate organized and dynamic actin structures in an in vivo system.

P962/B84

Roles for CIN85, an SH3 Domain- and Proline-rich Motif-containing Adaptor Protein, in Actin Assembly at Sites of Clathrin-mediated Endocytosis in Human Stem Cells.

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Clathrin-mediated endocytosis (CME) is highly conserved in eukaryotic cells and generally is augmented by a burst of actin assembly. In yeast cells, actin filament assembly plays an indispensable role in plasma membrane invagination and vesicle scission during this process. Proteins containing Src homology 3 (SH3) domains and proline-rich motifs (PRMs) are highly enriched among endocytic proteins. Interactions between these domains are important for precise special and temporal regulation of actin assembly at clathrin-coated pits (CCPs). SH3 domains of the endocytic adaptor protein Sla1 and the PRM of the coat protein Pan1 link actin assembly to endocytic sites by recruiting SH3- and PRM-containing proteins that play roles in the subsequent actin assembly step, including Las17, a WASP-related nucleation promoting factor (NPF). In mammalian cells, the functions and regulation of actin assembly during CME are less well understood. CME is a complex and highly regulated process, which involves the sequential recruitment of over 60 proteins to endocytic sites. Our lab studies CME in human induced pluripotent stem (iPS) cells using genome editing to express fluorescent protein fusions of endocytic proteins at endogenous levels, thus avoiding disruption of the physiological temporal and spatial organization of the process that can result from protein overexpression. In this study, to investigate actin assembly regulation during mammalian CME, we generated cell lines expressing Halotag labeled endogenous CIN85 (Sla1 homolog) using the CRISPR-Cas9 genome-editing. By applying total internal reflection fluorescence (TIRF) live-cell imaging, we quantitatively analyzed the dynamics of these proteins at CCPs. To our surprise, we observed distinctive behaviors of CIN85, which contains both SH3 domains and PRM, from its yeast homolog. To determine its functions in mammalian CME, we studied actin assembly and overall CME dynamics in the CIN85 knockdown or overexpressed cells and further analyzed the behaviors of various forms of SH3 or PRM mutants of CIN85. Our results provide important insights into roles for CIN85 in actin assembly at CCPs in human stem cells.

P963/B85

The Scaffold Protein RACK1 Modulates Cytoskeletal Rearrangement during Regulated Secretion.

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RACK1 (receptor for activated protein C kinase 1) is crucial for cellular activities and acts as a signaling hub in immune cell responses. Mast cell (MC) activation via antigen results in secretory granule release and is accompanied by cytoskeletal reorganization. The involvement of RACK1 in this process is unknown. By immunostaining, RACK1 was distributed throughout the cytoplasm of RBL-2H3 MCs. In antigen-stimulated cells, RACK1 was primarily localized adjacent to the plasma membrane. In order to investigate the relationship of RACK1 to the cytoskeleton in MCs, RACK1 was knocked down using lentiviral particles encoding shRNAs. Non-stimulated ShControl MCs were fusiform with a continuous

cortical F-actin layer and organized vimentin filament bundles. However, ShRNA mediated RACK1 knockdown (KD) MCs were rounded and the cortical F-actin was fragmented. The intermediate filaments in the cortical region in the KD MCs were also discontinuous. After antigen-stimulation, cortical F-actin was dramatically reduced, and the vimentin filament bundles were disorganized with vimentin free spaces in RACK1 KD MCs. When live LifeAct-RFP transfected MCs were analyzed by TIRFM, this disorganization of the cortical F-actin following stimulation in RACK1 KD MCs was highlighted. The distribution of microtubules in RACK1 KD MCs appeared to be unchanged. The microtubules continued to follow the cell shape. Although, co-immunoprecipitation experiments demonstrated an antigen-stimulation dependent increase in RACK1 interaction with cytoskeletal constituent proteins, high resolution confocal microscopy showed that there was a gap between RACK1 and actin. This suggests that RACK1 and actin are coupled through a linker and do not directly interact. Furthermore, RACK1 depletion significantly increased basal and stimulus-induced degranulation. $CD63^+$ secretory granules were localized in F-actin and vimentin-free cortical regions in non-stimulated RACK1 KD MCs and following antigen-stimulation they were concentrated in the apical region. This localization most likely facilitates and accelerates their release in RACK1 KD MCs. In contrast, RACK1 depletion resulted in a decrease in the secretion of newly synthesized mediators but did not affect newly formed mediator release. These results show that RACK1 is a critical regulator of actin dynamics and intermediate filament organization and provide additional information for understanding the molecular mechanisms involved in secretory granule release.

P964/B86

Role of the Actin Severing Factors, ADF/cofilin and Gelsolin, in Regulating the Fast Amoeboid Or Leader Bleb-based Migration of Cancer Cells.

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The cortical actomyosin cytoskeleton lies directly underneath the plasma membrane of all cells. In cancer cells, a high level of cortical actomyosin contractility is correlated with invasiveness. Recently, it was discovered that cancer cells can undergo fast amoeboid or Leader Bleb-Based Migration (LBBM), which requires the formation of a large and stable bleb for migration. Contained within all leader blebs, is a fast cortical actomyosin flow driving the cell forward, however the fundamental mechanisms required to maintain contractility and actin flow in leader blebs is not understood. Here, we hypothesize that actin severing factors, such as the ADF/cofilin family and gelsolin, are essential to maintain both contractility and actin flow for LBBM. Using RNAi in melanoma A375-M2 cells and a flow cytometry-based method for measuring F-actin, we find that co-depleting ADF and cofilin and not gelsolin led to a large increase in the level of F-actin, suggesting that ADF and cofilin together regulate actin in these cells. Moreover, using barbed-end assays and high-resolution imaging, RNAi of ADF and cofilin increased the number of cortical, polymerization competent, barbed-ends. Therefore, severing by these proteins appears to promote cortical actin turnover in melanoma A375-M2 cells. Furthermore, actin severing has been shown to promote contractility through the regulation of actin architecture. In line with this concept, RNAi of ADF and cofilin significantly increased cell deformability, as determined by a gel sandwich approach. As LBBM is stimulated by cell confinement, we next used a PDMS slab-based approach, which uses micron-sized beads for the precise control of cell confinement, to evaluate the role of ADF/cofilin in regulating cortical actin dynamics in blebbing cells. Ratio imaging of EGFP-cofilin and mScarlet revealed cofilin to be enriched within leader blebs, whereas RNAi of ADF and cofilin reduced bleb sizes and the frequency of motile cells. Strikingly, many blebs failed to retract in the

absence of ADF and cofilin, these cells had exceptionally long necks separating blebs and the cell body. Therefore, actin severing by these proteins appears to be critical to the function of the contractile bleb neck. Collectively, our data identifies ADF and cofilin as cortical actin remodeling factors required for the amoeboid migration of metastatic cancer cells.

P965/B87

Mechanistic Contributions of Coronin 1b and Coronin 1c in Cell Migration.

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Cell migration plays an essential role in physiological events including morphogenesis, wound healing and immune responses. To facilitate cell migration, actin must undergo rapid cycles of assembly and disassembly in a highly-coordinated manner. Type I coronins, a conserved class of actin binding proteins, have been shown to regulate the process of cell migration by modulating the turnover of the branched actin network at the leading edge. Recent work suggests that Coronin 1C (Coro1C) mediates the formation of membrane protrusions during cell migration; while the structurally similar Coronin 1B (Coro1B) regulates the disassembly of branched actin networks within these protrusions. However, despite these results our understanding of the function of Coro1B and Coro1C to actin dynamics and specifically in directed cell migration remains incomplete. Therefore, the overall objective is to elucidate the mechanistic contributions of Coro1B and Coro1C in branch stability and de-branching during migration. *I hypothesize that Coro1B and Coro1C regulate branched actin dynamics by modulating the functions of cofilin and actomyosin contractility during directed cell migration. To* test this hypothesis, I have established a fibroblast cell line derived from Coro1B knockout (KO), Coro1CFL/FL mice that is rescued with a flox-able Coro1B-GFP expression construct. Upon Cre-mediated recombination, both exogenous Coro1B-GFP and endogenous Coro1C are deleted to create a matched-pair null cell line. To assess the functional role of Coro1B and Coro1C in haptotaxis, I utilized a microfabrication technique that generates substrate-bound gradients. In addition, we used live-cell imaging and acute pharmacological inhibition of branched actin to examine changes to cell morphology, cell behavior, and cytoskeletal structure and organization in the matched-pairs. In the absence of Coro1B and Coro1C, cells lack directionality, migrate more slowly and have altered protrusions dynamics as compared to WT counterparts. Furthermore, deletion of Coro1B and Coro1C increased actomyosin contractility as well as increased total cofilin levels while inactive cofilin levels remained the same. These data suggest that Coro1B and Coro1C are highly involved in regulating actin dynamics at the leading edge which is required for effective actin remodeling and organization as well as directed migration.

P966/B88

Effects of Hiv-1 Tat on Microglial Phagocytic Activity and Cytoskeletal Protein Cofilin.

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Neuroinflammation is a mechanism that occurs in response to injury or infection and often its effects can lead to neurodegeneration. The neuroinflammatory process is implicated in a number of neurodegenerative diseases including Alzheimer's Disease (AD), Parkinson's Disease (PD), and HIV associated neurocognitive disorders (HAND). A key component of neuroinflammation are microglia that function to balance the micro-environment of neurons through the phagocytosis of bacteria, viruses and any foreign substances. Key findings from our studies have shown that the HIV-1 tat protein used as an inflammatory mediator induces a significant increase in two chemokines that directly control cell

migration and phagocytic activity. It is important to note that actin cytoskeleton plays an essential role in the homeostasis and normal functioning of cells, including microglia. Specifically, the motility, phagocytic activity, morphology and immune response of microglia hinges on the remodeling of the cystoskeleton. Over activated microglia can lead to alterations in their cytoskeleton thereby effecting functional activity. Cofilin and Phospho-Cofilin (P-Cofilin) are regulatory proteins that assist in actin reorganization and cytoskeletal function. We used HIV-Tat protein as a model inflammatory mediator to observe how microglial cells respond to an inflammatory environment. Previous research has been inconclusive in addressing these specific changes, therefore we aim to understand the effect inflammatory mediators have on microglia phagocytic activity and cytoskeletal structure. Through a series of cell culture experiments utilizing phagocytosis assays, immunofluorescence and immunoblotting techniques we have shown that microglia phagocytic activity is consistently decreased when exposed to inflammatory mediators and that modulation occurs as cytoskeletal protein activation decreases. Overall, our studies indicate that neuroinflammation can modulate microglial cytoskeletal proteins and function that could play a role in indirect neurodegeneration.

P967/B89

Ecm29-dependent Localization of the Proteasome Regulates Actin Polymerization and Immune Synapse Formation in B Cells.

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Recognition of surface-tethered antigens by the B cell receptor (BCR), leads to the formation of a specialized platform termed immune synapse (IS), where actin remodeling, signaling and membrane trafficking are focused. Concomitantly, BCR engagement triggers the recruitment of lysosomes to the IS by mobilizing the centrosome, a process which relies on the depletion of perinuclear actin. We previously described that the proteasome localizes at the centrosome and IS, and its activity is necessary for actin remodeling during IS formation. In this work we studied the cellular mechanisms involved in the localization of the proteasome and focused on Ecm29, previously shown to link the proteasome to motor proteins in neurons. We asked whether Ecm29 could mediate the localization of the proteasome to the centrosome and IS in B cells and evaluated whether silencing of Ecm29 could have an impact in actin remodeling within these sites. We first characterized proteasome dynamics at the IS in B cells activated with immobilized antigens, by Total Internal Reflection Fluorescence Microscopy (TIRFM). For this, we labeled microtubules or the actin cytoskeleton together with the proteasome, using a specific probe, which allowed us to track the proteasome in live cells, upon activation. We found that proteasome movements at the IS followed the microtubule network and its localization was coupled to local actin depletion. Additionally, our results show that Ecm29-silenced B cells display reduced proteasome mass and activity at the centrosome, which was more enriched at the synaptic membrane, compared to control cells. In agreement with these observations, actin dynamics at the IS of Ecm29-silenced B cells was increased, as measured by the Fluorescence Recovery After Photobleaching (FRAP) of Actin-GFP. Moreover, actin accumulation within isolated synaptic membranes was reduced and BCR signaling was enhanced in Ecm29-silenced cells. Thus, Ecm29 controls the localization of the proteasome to the IS, where it mediates local actin remodeling and B cell activation. Finally, we determined the effect of Ecm29 silencing on the centrosome pool of actin and found that the perinuclear actin clearance, triggered by BCR activation, was delayed. Consequently, lysosome and centrosome

recruitment to the IS were impaired in Ecm29 silenced B cells, which also displayed deficient antigen extraction and presentation. In conclusion, we show that Ecm29 regulates the localization of the proteasome in B cells, where it tunes actin dynamics at the IS and promotes the depletion of actin at the centrosome, controlling activation, antigen extraction and presentation in B cells.

P968/B90

Both Branched and Bundled Actin Are Required for B-cell Receptor-induced B-cell Response to Antigen.

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B-cells confer adaptive immunity through generating antibodies. Upon antigen recognition by the B-cell receptor (BCR), B-cells spread over the antigen-presenting surface, which promotes BCR aggregation into microclusters, amplifying signaling. Following spreading, the B-cell contracts, driving BCR microclusters to reorganize into a large central BCR cluster, which leads to signal attenuation. Inhibition of B-cell spreading or contraction through perturbing actin results in immune deficiency and autoantibody production in mouse models. However, how actin reorganization transitions B-cells from spreading to contraction remains unclear. Here we examined the role of branched and bundled actin, the two major types of actin structures, in this process. We utilized Arp2/3 and formin inhibitors to perturb the generation of branched and bundled actin. We analyzed their effects on lifeact-GFP expressing primary B-cells activated by antigen presented on planar lipid bilayers using live cell imaging by total internal reflection fluorescence (TIRF) microscopy. Perturbation of either branched or bundled actin before B-cell spreading reduced the overall level of F-actin accumulation at the B-cell contact zone. Particularly, perturbation of bundle actin disrupted the centripetal flow of actin. Concurrently, the rate of B-cell spreading was reduced and B-cell contraction was inhibited, which led to a decrease in BCR aggregation. Perturbation of branched actin after spreading inhibited B-cell contraction. Consequently, BCR clusters failed to move centripetally and grow in size. Our results suggest that both branched and bundled actin are required for not only B-cell spreading, but also B-cell contraction, which regulates BCR signaling by modulating the formation, growth and centripetal movement of BCR clusters.

P969/B91

Actin Dynamics and Organization at the T Cell Immune Synapse: the Role of the Signaling Protein Bcl10.

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T cells form an essential part of the adaptive immune system. Stimulation of the T cell receptor (TCR) leads to rapid remodeling of the actin cytoskeleton. Defects in actin dynamics and organization lead to impaired immune response. Here, we use total internal reflection fluorescence (TIRF) microscopy to monitor actin dynamics at the immune synapse of primary murine effector T cells. Quantification of these dynamics using spatiotemporal image correlation spectroscopy (STICS) reveals two distinct temporal phases, showing differences in speeds and directionality. Interestingly, our results indicate that cells lacking Bcl10 - a protein involved in NF-κB signaling - display faster actin flows, more dynamic lamellipodia and smaller actin rings, resulting in altered T cell function.

P970/B92

Abelson Tyrosine Kinases Link Cbl E3 Ubiquitin Ligase to Regulate WAVE Regulatory Complex.

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The family of Abl-interactor (Abi) proteins is a downstream target of Abelson (Abl) tyrosine kinases and a component of WAVE regulatory complex (WRC). As a subunit of WRC, Abi proteins interact with diverse membrane proteins and intracellular signaling molecules and link them to the actin cytoskeleton remodeling. The ability to interact with the regulatory machinery of actin assembly as well as membrane proteins places Abi1 at a central position in the network that controls endocytosis and/or intracellular trafficking of membrane receptors. However, the mechanism by which Abi proteins regulates these cellular processes remains largely unknown. We have previously shown that Abi2 is rapidly degraded in Bcr-Abl-positive leukemic cells through an ubiquitin-dependent proteolysis pathway. In this study we present the evidence that Bcr-Abl-induced Abi2 degradation is mediated by Cbl E3 ubiquitin ligases. Double knockout of c-Cbl and Cbl B in Bcr-Abl-transformed leukemic cells completely abolishes Bcr-Abl-induced degradation of Abi2. We have identified a Cbl-PKB binding motif in Abi2 and we show here that the tyrosine 213 (Y213) in this motif is essential for Cbl-mediated degradation. A mutation of Y213 to phenylalanine in Abi2 is sufficient to abrogate the Bcr-Abl-induced degradation of Abi2. Together, these studies uncover a novel mechanism by which the WRC is regulated in leukemic cells. Given that both Cbl and WRC have been shown to regulate membrane receptors, these studies may shed light on the mechanism that controls membrane receptor endocytosis and trafficking.

P971/B93

Analysis of the Proteome of the Immunological Synapse in NK Cells.

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NK cells are innate immune cells that provide protection against virally compromised or transformed cells. Direct killing occurs through the release of lytic granules onto the target cell at the immunological synapse (IS). The IS is a unique structure formed at the site of contact between the immune cell and the target cell and is characterized by a dense actin mesh on the effector cell side. The formation of dynamic hypodense regions of actin in this mesh allows for lytic granules to reach the plasma membrane, resulting in the release of the granule's contents within the IS space. While many key components regulating the dynamics of the IS have been identified, its complete composition remains unknown and our understanding of this structure remains therefore limited to the list of known targets with available antibodies. To address this, we sought to create an innovative experimental approach to identify the molecular components mobilized on the NK cell side of the IS during cytotoxic activity that would allow the identification of new components. We have developed a method to extract and purify the portion of the NK cell cortex engaged in the IS. Ligand-coated beads replaced typical target cells and were incubated with a human NK cell line (NK-92), allowing the IS to form. Using a combination of cross-linkers, detergent and sonication, we then separated and purified the cellular cortex at the IS from the bulk of the NK cell. To demonstrate that our technique works, we screened by Western blot for a series

of known markers for the IS and other various cellular compartments to testify to the specificity and efficacy of our purification approach. In parallel, we observed by immunofluorescence that F-actin and myosin IIa are maintained as a patch on the beads following sonication of the whole cell. The footprint left by the effector cell onto the bead suggests that the architecture of the IS is preserved by our extraction protocol, increasing the probability of purifying intact cortices. In a subsequent large-scale assay, we have analyzed by mass spectrometry the proteome ("immune synapsome") present at the IS and compared the activated and tethered-only NK cell conditions. 515 proteins were unique to the cortex of activated NK cells and 256 additional ones were enriched compared to those in the tethered-only cortex condition. Among the proteins enriched, we confirmed the identification of expected IS-localized proteins such as CD3ζ, ZAP70, granzymes, perforin, and several downstream adaptors of the activation receptor in the activated NK cell cortex sample. This result validates our method and provides confidence to further investigate other IS-enriched proteins, with the ultimate goal of uncovering novel key regulators of NK cell cytotoxic function.

P972/B94

Novel Actin Ring Structure in *Clamydomonas in* Vegetative *Clamydomonas*Cells Plays Potential Role in Nuclear Integrity.

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The unicellular, green alga *Chlamydomonas reinhardtii* is an excellent model organism to study the cytoskeletal actin network because of its two actin genes, *IDA5* and *NAP1*. IDA5 is a conventional actin that is about 90% identical to mammalian actin, whereas the divergent actin NAP1 has only about 65% sequence identity to mammalian actin. Using a phalloidin staining protocol, we are able to visualize the complex branched actin network. Upon treatment with Latrunculin B, a drug that depolymerizes IDA5, NAP1 is upregulated and a novel ring-like structure made of filamentous actin forms around the nucleus in about 60% of wild-type cells. Interestingly, the same treatment in gametes results in significantly less rings. These rings can also be seen in *ida5* null mutants, but not in *nap1* null mutants, suggesting NAP1 is necessary and sufficient for ring formation. Quantification of these perinuclear actin rings shows that ring area and perimeter scale with cell volume and surface area. The function of these rings remains unknown, but we have observed possible DNA leakage from the nucleus during the time of early ring formation leading us to hypothesize that these ring structures play a critical role in nuclear integrity.

P973/B95

Unveiling the Role of Actin Regulation on Kras-induced Autophagy Using *Caenorhabditis Elegans* as an in *Vivo* Model.

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KRAS is a small GTPase protein that is found mutated in 30-50% of sporadic colorectal cancer (CRC). Cancer-related mutations lead to activated KRAS and have been shown to upregulate autophagy. Moreover, it was shown that KRAS activating mutations lead to an increase in the levels of activated Rho and Rac, two key regulators of actin dynamics. We are interested in understanding whether different actin regulators are involved in RAS-induced autophagy. The RAS signalling pathway has been primarily dissected in the *C. elegans* vulva. LET-60 (RAS homologue in *C. elegans*) is required for vulva development, which involves tightly controlled cell proliferation and differentiation. Expression of the

activating mutant LET-60(G13E) induces ectopic vulva cell divisions, migrations and fusions resulting in a multivulva phenotype, indicative of an excess of proliferative signaling. As vulva development is thoroughly understood and fluorescent tools are available to directly monitor it by microscopy, *C. elegans* vulva development is a unique model to explore the mechanisms underlying the interplay between RAS, actin dynamics and autophagy in the carcinogenic process. We are using depletion of LET-60, autophagy machinery and actin regulators by RNAi, generation of let-60 activating mutants by CRISPR/Cas9 technology, and live imaging-based approaches to assess vulva development and autophagy. We confirmed that animals in which LET-60(G13E) is the only source of LET-60 do present more than one vulva. Moreover, by expressing LGG-1::GFP (equivalent to LC3) in these animals we were able to prove that an increase of autophagy is also observed in these animals. Our analysis indicates that specific actin regulators are involved in Ras-induced autophagy, as depletion of LET-60 or several actin regulators leads to decreased levels of autophagy in the vulva of let-60(G13E) animals. Further studies will be necessary to dissect how the LET-60 pathway regulates actin dynamics to drive autophagy.

P974/B96

Enhancing Sensitivity of Glioblastomas to Ionizing Radiation by Actin and Tubulin Cytoskeleton Disruption.

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Glioblastomas (GBM) are a common primary brain tumor in adults of high malignancy characterized for increased cellular proliferation and resistance to chemo and radiotherapy. The cytoskeleton of GBM is closely related to its high capacity of invasiveness and migration, but little is known about the role of cytoskeleton in DNA damage response (DDR) and repair pathways, or its possible involvement in therapies resistance. The main objective of this study is to evaluate whether the response of glioblastoma cell lines to ionizing radiation (IR) is altered or enhanced as actin cytoskeleton or microtubules are somehow perturbed. It also aims to verify if cytoskeleton disruption affects the DNA damage response pathway and thereafter DNA repair, and whether this mechanism is p53-dependent or not. The GBM cell lines U87-MG (p53 wild type) and T98G (p53 mutated) were used as experimental models. The actin cytoskeleton was destabilized with Cytochalasin D (Cyto D), and the microtubules with Vincristine, and then cells were exposed to gamma radiation through a Cobalt-60 irradiator. Cell proliferation curves showed different behaviors between these two cell lines. T98G was more sensitive to IR than U87-MG, but the combined treatment Cyto D + IR lead to an expressive reduction in proliferation only in U87-MG cells. Clonogenic assays confirmed an additive effect of Cyto D and IR, decreasing the survival of T98G cells, while for U87-MG the effect was synergistic, with a higher increase in the cell's sensitivity to IR. On the other hand, microtubules destabilization provoked the same response in both cell lines. Vincristine treatment enhanced significantly the sensitivity to IR with synergic effects, as evidenced by an expressive decrease of cellular proliferation and survival in both cell lines. Immunofluorescence assays showed that γH2AX foci formation after IR is strongly inhibited in U87-MG under previous Cyto D treatment, but this combined treatment only slightly reduced the signaling in T98G. Vincristine + IR association changed the kinetics of H2AX phosphorylation and also provoked an inhibition in this signaling in both cells, but especially in T98G. These results indicate that Cyto D enhanced significantly the sensitivity of U87-MG therefore suggesting a dependency on p53 activity. Otherwise, the effect of microtubules disruption by Vincristine in the response to IR was similar for both

cells, seemingly independent of p53 activity. Both cytoskeleton components seem to be essential for DDR and DNA repair pathways, however through the regulation of different mechanisms.

P975/B97

Absent Actin Filament Regulation Reinforces Pathologic Acetylcholine Receptor Clustering.

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Background: NUP88 is a protein of the nuclear pore complex, the main transportation hub in the cell for exchange between the cytoplasm and the nucleus. We showed earlier that biallelic mutations in *NUP88* lead to a rare condition, termed fetal akinesia deformation sequence, that is characterized by reduced fetal movement (fetal akinesia). Known genetic causes for fetal akinesia are mutations in genes linked to signaling at the neuromuscular junction (NMJ), such as *RAPSN* or *MUSK*. Objective: We sought to identify cellular mechanism(s) in the pathogenesis of fetal akinesia disorders. Herein, we established two specific aims: first, characterizing the overall interplay of the cytoskeleton in fetal akinesia and second, disclosing the function of NUP88 in muscle cell development in health and in NUP88-related fetal akinesia. Results: Firstly, by using micropatterning and bioinformatic tools, we extensively analyzed cytoskeletal components of fibroblasts from fetal akinesia patients. We found that these cells suffer from abnormal contractile actin-myosin modules and enhanced focal adhesion maturation due to increased activation of RhoGTPase. Over-representation of actin microfilaments also perturbed the microtubular and intermediate filament network in these cells. Secondly, by using murine myoblasts, we could show that loss of Nup88 impairs muscle cell differentiation and furthermore, altered acetylcholine receptor (AChR) clustering in *vitro* at the plasma membrane in murine myotubes and in *vivo* in skeletal muscle of zebrafish. The mechanism of AChR patterning involves podosomes, conical actin-enriched structures at the plasma membrane. Consecutively, podosomal structure and number were increased in Nup88-mutant myotubes and in fetal akinesia fibroblasts. Conclusion: Our results establish a consequential link between dysregulation of the actin cytoskeleton, muscle cell maturation, and AChR patterning at NMJs. Intact NMJs are critical for proper skeletal muscle contraction and especially for fetal movement. Thus, fetal akinesia disorders are the result of pathologic actin microfilament regulation involving proteins responsible for signaling at NMJs and most interestingly, the nuclear pore complex protein NUP88.

P976/B98

Physiological Concentrations of Hydrogen Peroxide and Glutamate, Induce Tunneling Nanotube Formation in Hela and Cad Cells.

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Discovered in 2004 tunneling nanotubes (TNTs) are bridge like structures that allow cell-to-cell communication. Initially described as long, actin-based, non-adherent to the substratum, transient nano-structures, their main function is the intercellular transport of cellular components, electrical signals and pathogens. Thus, TNTs have been implicated in viral propagation, neurodegenerative diseases, and cancer. Most research has focused on examining the molecules within cells that aid in TNT formation and function, but little has been documented on the role that exogenous molecules have on the formation of TNTs. Past research has determined that hydrogen peroxide (H_2O_2)—coincidentally secreted by cancer cells—induces TNTs in various cell lines; however, the amount of H_2O_2 used was

above normal physiological concentrations. The aim of this study was to establish if TNT formation can be induced using physiological H_2O_2 concentrations, as well as determine if TNT formation can be induced exogenously using H_2O_2. In addition, we also investigated, glutamate, another important signaling molecule released by cancer cells and an inducer of actin based filopodia in astrocytes, as a possible tunneling nanotube inducer. The data obtained through the use of fluorescence microcopy suggests that physiological concentration of H_2O_2 and glutamate are able to increase the formation of TNTs in both HeLa cells and the neuronal CAD cell line. Moreover, preliminary data obtained with the use of a microscope capillary single cell sorting system suggests that high concentrations of H_2O_2 can induce the formation as well as the elongation of possible preliminary TNT structures in distances ranging from 20-65 µm. Altogether, these results provide useful insights regarding TNT formation pathways in cells as well as the exogenous molecules involved in the formation of TNTs.

P977/B99

CD13 Is a Critical Regulator of Cell-cell Communication Via Tunneling Nanotubes.

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CD13 is a Critical Regulator of Cell-Cell Communication via Tunneling Nanotubes Emily Meredith, Mallika Ghosh and Linda H. Shapiro Center for Vascular Biology, University of Connecticut Health Center, Farmington, Connecticut Cell-cell communication is essential for proper growth, function and survival of organisms. Tunneling Nanotubes (TNTs) are open ended actin based cellular protrusions detached from the substratum that connect two or more distant cells and facilitate cell-cell communication by transporting cargoes ranging from cytoplasmic proteins to intact organelles. However, little is known about the molecular mechanisms that induce and maintain TNT formation, control the direction of their growth or regulate cargo transfer under physiologic or pathologic conditions. Recently, we have discovered that adhesion molecule, CD13, is a physiologic regulator of TNT formation in a variety of endothelial and myeloid cells. Indeed, cells stressed by low serum conditions led to robust TNT formation in the wild type human Kaposi sarcoma endothelial cell line (KS) which was clearly absent in KS-$CD13^{KO}$ CRISPR lines lacking CD13. Moreover, treatment of WT KS cells with a CD13 activating antibody induced TNT formation in a CD13-dependent manner. Interestingly, CD13 and actin are highly abundant at the base as well as along the length of the TNTs, potentially indicating a role for CD13-mediated membrane and protein organization at the site of TNT initiation. Homotypic coculture of WT and $CD13^{KO}$ cells indicated that CD13 expression is necessary for membrane and organelle transfer via functional TNTs. Mechanistically, treatment with peptides designed to interfere with CD13's cytoplasmic signaling domain diminished TNT formation in a dose dependent manner, suggesting that CD13-dependent signal transduction mediates TNT formation. Future investigation will focus on the identification of fundamental mechanisms and molecules involved in transfer of organelles through TNTs in a CD13 dependent manner.

P978/B100

The Desmosomal Cadherin Desmoglein-2 Controls Rap1 GTPase-mediated Cell Spreading Independent of Cell-cell Adhesion.

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The desmosome is a cell-cell adhesion complex which facilitates the mechanical stability of tissues and cell-cell communication. Desmosome function depends upon a tripartite organizational structure wherein transmembrane cadherins (Desmoglein and Desmocollin) link adjacent cellsin the extracellular space, armadillo proteins (Plakophilin and Plakoglobin) stabilize the intracellular plaque, and the cytolinker Desmoplakin (DP) connects the plaque to the intermediate filament network. In addition to their central role in maintaining cell-cell junction integrity, desmosomal cadherins also coordinate biological processes such as proliferation, apoptosis, differentiation and cell migration. In our study, we sought to investigate the signaling mechanisms involved in control of actin architecture via desmosomal cadherins, using A431 cells lacking either Desmoglein-2 or Desmocollin-2, generated via CRISPR-mediated knock-out (Dsg2 KO and Dsc2 KO, kindly provided by Dr. Daniel Conway, VCU). Wildtype, Dsg2 KO and Dsc2 KO A431 cells were subjected to single cell spreading assays on different extracellular matrix proteins. Compared to control cells, Dsg2 KO cells displayed a significant increase in spreading area on both fibronectin and collagen. Interestingly, knockout of Dsc2 caused the opposite effect, leading to a significant reduction in spreading area. As these experiments were performed in singly spreading cells, these experiments have identified a novel cell-autonomous, cell-cell adhesion independent role for desmosomal cadherins in regulation of cell spreading. Spreading changes in Dsg2 KO cells were dependent on Rap1 GTPase, as siRNA-mediated knockdown of Rap1 in Dsg2 KO cells rescued the increase in spreading area. Dsg2 KO cells also demonstrated a decrease in expression of Paxillin, suggesting that the enhanced spreading phenotype may be due to alterations in cell-matrix adhesion. Not surprisingly, loss of Dsg2 results in a significant increase in the Triton-X solubility of other desmosomal components such as Plakophilin-2, Plakophilin-3, Plakoglobin, and Desmoplakin. Knockdown of Desmoplakin was able to rescue the enhanced spreading seen in Dsg2 KO cells alone, thereby implicating Desmoplakin-dependent signaling in control of cell spreading via Dsg2. Future experiments will investigate the upstream signaling mechanisms via which Dsg2 and Desmoplakin regulate Rap1 activity and signaling. These data have identified a novel cell-cell adhesion independent role for Desmoglein-2 in mediating cell spreading via Rap1, which provides significant insight into the signaling mechanisms via which desmosomal cadherins control cell-matrix attachment and cytoskeletal architecture.

Actin-membrane interactions

P979/B101

In Vivo Knockdown of the IP3R1 Calcium Channel Disrupts Er-tbc Membrane Contacts and Alters the Actin Cytoskeleton in Rat Sertoli Cells.

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During spermatogenesis, late stage spermatids are temporarily attached to the apex of Sertoli cells in the seminiferous epithelium by large anchoring junctions. These large junction complexes are termed ‘ectoplasmic specializations’ (ESs) and are proposed to be disassembled by clathrin based endocytic structures only found in mammalian Sertoli cells, called ‘tubulobulbar complexes’ (TBCs). TBCs have a dendritic actin network encircling their tubular portions that extend from the plasma membrane to the ‘bulb’ where the actin is replaced by a cisternae of ectoplasmic reticulum (ER) that forms a membrane contact site (MCS) with the TBC. Previously, we’ve localized the IP3R calcium channel to the TBC bulb-ER contacts as well as other calcium exchange machinery to various parts of the ER in the apical process of Sertoli cells. The movement of calcium between membranes at MCSs is now a well-established function

of MCSs, and allows for substantial alteration to target organelles such as in mediating the fusion and acidification of endosomes. Changes in local calcium levels can also affect the polymerization of actin networks in cells generally. We've proposed that the presence of IP3R1 at TBC-ER contacts may indicate that calcium exchange is a function of these contacts and that this exchange could facilitate the maturation and fusion of TBC bulbs as they develop into putative endosomes or alter local actin networks. If calcium exchange from the ER is responsible for regulating the maturation of TBC bulbs in the apical process or altering actin networks, then knocking down the IP3R1 should alter TBC bulb morphology and distribution, disturb the ER-TBC contacts, and change the actin networks in Sertoli cells. To test this, we injected the testes of Sprague Dawley rats with siRNAs against IP3R1 (ITPR1) and collected tissues at 2 days post injection and processed the tissues for either western blot, immunofluorescence, or electron microscopy. Tissues injected with siRNA against the gene associated with IP3R1 (ITPR1) showed significant morphological alterations to the actin networks including a loss of TBC actin and the acquired presence of ectopic para-crystalline actin bundles in Sertoli cell stalks. There was also a change in the abundance and distribution of a known TBC-ER MCS specific marker, ORP9. Together, these findings are consistent with the hypothesis that calcium exchange at TBC-ER contacts is involved in regulating actin dynamics at TBCs and in maintaining the structure of TBC bulbs.

P980/B102

Identifying Components of the Molecular Clutch That Regulates Apical Constriction.

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Apical constriction is mechanism commonly used to change a cell's shape and initiate morphogenesis in a broad range of animal systems, including in vertebrate neural tube formation. Apical constriction occurs as a result of actomyosin contractions at the apical surface, which shrink the exterior face of a cell, or cells, bending or internalizing a cell or tissue. In the model system *C. elegans*, gastrulation begins with two endodermal precursor cells that utilize apical constriction to internalize at the 26-28 cell stage. Surprisingly, data suggests that a temporally regulated link is required for the actively contracting apical actomyosin networks to pull the apical cell-cell junctions inward, triggering apical constriction. We refer to this mechanism as the clutch. We used single-cell RNA-seq data to identify genes of interest and this approach has identified a candidate that functions at least in part in this clutch mechanism - *zyx-1*/zyxin. Zyxin is part of a family of LIM domain-containing proteins and has known functions in other systems consistent with a clutch-type mechanism. *C. elegans* embryos lacking zyxin display gastrulation defects and have defective coupling during apical constriction. Using a similar RNA-seq approach, we have identified other LIM domain-containing genes that are specifically expressed in other internalizing cells. LIM domain-containing proteins, including zyxin, are broadly conserved, and these results could apply to mechanisms that regulate apical constriction in other systems.

P981/B103

Investigating the Role of a Conserved Amphipathic Helix in Curvature Sensing by the Septin Cytoskeleton.

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Cell shape is often described in terms of membrane curvature. Septins are filament forming, GTP-binding proteins that assemble on positive, micrometer-scale curvatures from yeast through humans. At

these sites, septins function in several cellular processes including cytokinesis, migration, and autophagy. However, the mechanism for curvature sensing by septins is not fully understood. Recently, we identified an amphipathic helix (AH) at the very C-terminus of the budding yeast septin Cdc12. This AH domain is highly conserved and is both necessary and sufficient for septins to sense membrane curvature. However, it is currently unclear how the AH domain enables septins to preferentially adsorb onto micrometer-scale membrane curvatures. An interesting feature of the septin complex is its symmetry across its long axis, with neighboring AH domains spaced ~24 nm apart. We wondered if we could change septin curvature preference by changing the space between AH domains within the septin complex. Wild-type septin complexes (AH spacing ~24 nm) preferentially assemble onto a membrane curvature of 2 μm^{-1}. In contrast, changing the spacing between AH domains from ~24 nm to ~16 nm, we observed preferential binding of septins onto a curvature of 4 μm^{-1}. Interestingly, by moving the AH domains 32 nm apart, we observed no difference in curvature preference compared to wild-type (preferred curvature = 2 μm^{-1}). These data suggest that the space between AH domains is an important factor in promoting septin assembly onto specific membrane curvatures.

P982/B104

Single-molecule Strategy Uncovers the Turnover of Actin and Coat Proteins during Clathrin-mediated Endocytosis.

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Actin dynamics generate forces to deform the membrane and overcome the cell's high turgor pressure during clathrin-mediated endocytosis in yeast, but precise molecular details are still unresolved. Our previous models predicted that actin filaments of the endocytic meshwork continually polymerize and disassemble, turning over multiple times during an endocytic event, similar to other actin systems. We applied single-molecule speckle tracking in live fission yeast to directly measure molecular turnover within endocytic sites for the first time. In contrast with the overall ~20-sec lifetimes of actin and actin-associated proteins in endocytic patches, we detected median single-molecule residence times around 1 to 2 sec, and similarly high turnover rates of membrane-associated proteins in CME. Furthermore, we find heterogeneous behaviors in many proteins' motions. These results indicate that the formation of endocytic vesicles is a highly dynamic process, and that endocytic proteins turn over up to 5 times during the formation of an endocytic vesicle. These new data forces us to revise quantitative models of force production by the actin machinery during endocytosis.

P983/B105

Mechanistic Insights into Actin-based Force Generation during Clathrin-mediated Endocytosis Via *in Situ* Cryo-Electron Tomography.

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Networks of interconnected actin filaments act in essential cellular processes, for example by providing force generated through polymerization. One of these processes is clathrin-mediated endocytosis (CME), a major pathway for down-regulation of signaling receptors and uptake of extracellular material, that involves a series of plasma membrane remodeling steps resulting in the formation of clathrin-coated vesicles (CCVs) containing cargo. Structural aspects of CME have been extensively studied by transmission electron microscopy of platinum replicas, and of thin sections of plastic embedded cells.

However, both of these sample preparation methods have been shown to alter actin cytoskeleton organization. As a result, precise structural information on actin organization in CME, which is key to understanding actin's mode of function in this process, is lacking. Here, we made use of recent technological advances in cryo-electron tomography (cryo-ET) to discover the 3-dimensional actin cytoskeleton organization in mammalian CME at near-native state conditions. Combined with mathematical modeling, this analysis promises to reveal how actin functions in CME. We found growth conditions for SK-MEL-2 cells that allow cryo-ET of intact cells. Strikingly, individual actin filaments, actin branches, CME sites and CCVs can clearly be identified in our tomography data. The actin cytoskeleton associated with CME sites and CCVs consists of branched as well as unbranched filaments. Filaments at CME sites are not exclusively localized around the neck of membrane invaginations as was seen before by platinum replica EM, but rather form more complex 3-dimensional arrangements. We determined filament polarity based on branch point geometry and found an accumulation of plus-ends at positions that would allow for force production by polymerization to support plasma membrane deformation and CCV transport. Moreover, branched filaments do not arise from a common single filament, contradicting the theory that a single mother filament gives rise to the entire CME-associated network. Surprisingly, we also found varying amounts of very long, heavily bent actin filaments at CME sites. Based on mathematical modeling, these filaments are predicted to store elastic energy, which augments force generation by actin polymerization to support membrane deformation and thus represents a previously unrecognized mode of actin-based force production in CME. Taken together, our results reveal the complex actin filament organization at endocytic sites in unprecedented detail. We are in the process of in-depth quantitative analysis of our data, which will, in combination with mathematical modeling, ultimately result in a detailed understanding of actin function in CME.

P984/B106

Force Patterning at the Immune Synapse.

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p { margin-bottom: 0.25cm; line-height: 115%; }a:link { } p { margin-bottom: 0.25cm; line-height: 115%; }a:link { } a crucial phase of immune response is antigen acquisition by B cells in the lymph node. This occurs through endocytosis of native antigen presented on the surface of opposing cells, via a specific cell-cell contact, the immunological synapse. We investigate the forces developed by B cell at the immunological synapse during antigen extraction. We found that two main types of force, a centripetal peripheral one and a central one perpendicular to the synapse, are essential for antigen extraction. The centripetal peripheral component is Myosin II dependent while the central forces are produced by actin-rich protrusions and surprisingly correlate with the antigen extraction spots. Both global energy and antigen extraction are strongly reduced by Myosin II inhibition and are increased, by lysosomal calcium release. The interplay between global and local forces dictated by the organization of the actomyosin cytoskeleton therefore controls endocytosis at the immune synapse.

P985/B107

Identification of a Novel B Cell Actomyosin Network That Promotes Antigen Contraction during Immune Synapse Formation.

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B cells are a critical branch of the immune system and drive antibody-based protection. The strength of an antibody response is determined by the ability of B cells to extract and internalize membrane-bound antigens from antigen-presenting cells (APCs). Antigen uptake requires the formation of an immune synapse (IS) where B cell receptor-bound antigens are moved centripetally into a central cluster before being extracted from the APC and internalized. The force provided by the actin motor myosin 2A (M2A) powers antigen extraction. B cells that lack M2A activate aberrantly and mount weak antibody responses. However, the organization of the actomyosin network, its role in IS formation, and the mechanism by which M2A powers antigen extraction in B cells are unknown. Here we test the hypothesis that the actomyosin network drives the events of IS formation that promote B cell activation and antigen uptake. We first define the dynamic organization of actin and M2A at the IS using the super-resolution imaging modalities TIRF/SIM, 3D-SIM and Airyscan. On functionalized glass and planar lipid bilayers, the A20 B cell line forms concentric arcs in the medial portion of the IS. These arcs are rich in M2A based on immunostaining, and on imaging cells in which endogenous M2A was tagged with GFP using CRISPR. 3D-SIM imaging of APCs with primary splenic B cells isolated from M2A-GFP knock-in mice shows that M2A polarizes towards the IS. B cell IS studies are often performed using antigen stimulation only. In *vivo*, B cell integrins provide adhesion to APCs and, via an unknown mechanism, allow for IS formation and antigen uptake with weakly-stimulating antigens. Surprisingly, we found that actomyosin arc formation in primary B cells requires both antigen and integrin costimulation, conditions that reflect physiological B cell activation. These contractile actomyosin arcs are especially prominent in primary B cells such that the actomyosin arcs are the major actin structure at the IS. The contractile nature of the actomyosin arcs that dominate at the primary B cell IS may explain why integrin costimulation boosts B cell responses to weakly-stimulating antigens. Notably, integrin costimulation on bilayers produces actin arcs that sweep peripheral antigen clusters centripetally and is required for contracting low amounts of antigen to form the IS. Moreover, M2A inhibition abrogates the organization of actin arcs and prevents antigen centralization. Therefore, we have identified in primary B cells a novel actomyosin network, which comprises the major actin structure at the IS and promotes robust antigen centralization during IS formation. Current efforts are directed at defining the mechanism by which the actomyosin network drives antigen extraction from APCs using live-cell volumetric imaging.

P986/B108

Structural and Mechanistic an alysis of Two Distinct Endocytoc Pathways for B Cell Receptors in B Lymphocytes.

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B lymphocytes play a critical role in adaptive immunity. Upon antigen binding, B cell receptors (BCR) cluster on the plasma membrane and are internalized by endocytosis. B cells capture diverse antigens in various contexts and concentrations. However, it is unclear whether the mechanism of BCR endocytosis changes in response to these factors. Here, we studied the concentration dependence of soluble anti-human IgM Fab'2-induced BCR clustering and internalization in the human IgM+ DG-75 B cell line using correlative super resolution fluorescence and electron microscopy. First, by directly visualizing

nanoscale structures associated with BCR clusters we provide evidence that BCR cluster size increases with Fab'2 concentration. Next, we directly show that the mechanism of internalization switches in response to BCR cluster size. Specifically, at low concentrations of Fab'2, B cells internalize small BCR clusters by classical clathrin-mediated endocytosis. At high Fab'2 concentrations when cluster size increases beyond the size of a single clathrin coated pit, B cells retrieve clathrin-capped BCR clusters using large invaginations of the plasma membrane that may require actin for internatlization. We propose that in B cells the mechanism of endocytosis switches to accommodate large plasma membrane receptor clusters induced when cells encounter high concentrations of antigen.

P987/B109

Self-organization and Load Adaptation by the Mammalian Endocytic Actin Network: Integrating Modeling with Experiment.

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Force generation due to actin assembly is a fundamental aspect of membrane sculpting for many essential processes. We hypothesized that the spatial restriction of actin filament nucleators to the base of the pit and actin/membrane linkages in the clathrin coat might dictate how endocytic actin networks organize, produce force, and adapt to elevated membrane tension. Here, we use a multiscale computational model constrained by experimental measurements to show that a minimal branched actin network is sufficient to internalize endocytic pits against physiological membrane tension. A parameter sweep identified the number of Arp2/3 complexes as particularly important for robust internalization, which prompted the development of a molecule-counting method in live mammalian cells. From engineered GFP-tagged multimeric proteins of defined copy number, we constructed an intracellular calibration curve relating fluorescence of endogenously GFP-tagged proteins to numbers of molecules in live cells. A peak of ~200 molecules of Arp2/3 complex assemble at endocytic sites marked by the clathrin adaptor AP2-RFP in human induced pluripotent stem cells. Our simulations also revealed that actin networks self-organize in a radial branched array with barbed filament ends oriented to grow toward the base of the pit, and that the distribution of linker proteins around the endocytic coat is critical for this organization. Surprisingly, our model predicted that long actin filaments bend from their attachment sites in the coat to the base of the pit and store elastic energy that can be harnessed to drive endocytosis. This prediction was validated using cryo-electron tomography on cells, which revealed the presence of bent actin filaments along the endocytic site. Furthermore, we predict that under elevated membrane tension, the self-organized actin network directs more growing filaments toward the base of the pit, increasing actin nucleation and bending for increased force production. Thus, our study reveals that spatially constrained actin filament assembly utilizes an adaptive mechanism that enables endocytosis under varying physical constraints. We anticipate that the principles of self-organization and load-adaptation uncovered in this study will apply to other cellular membrane-deforming processes driven by the cytoskeleton.

P988/B110

Intravital Subcellular and Single Molecule Imaging Reveal Multiple Actin Filament Populations Collaborate in the Remodelling of the Secretory Granule Membrane.

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The actin cytoskeleton contributes to many cell functions involving the remodelling of membranes. This is achieved both via interaction of actin filaments with myosin motors and through the force generated by branched actin networks. One of these processes is exocytosis of large secretory granules in the salivary gland of rodents. We used intravital subcellular microscopy (ISMic) to identify the types of actin filaments that are required for the granule integration into the apical membrane and intravital single molecule microscopy (iSiMM) to study the molecular behaviour and nanostructures at the basolateral membrane. We find at least two types of actin filaments are associated with exocytosis at the apical membrane. Actin filaments containing tropomyosin Tpm3.1 are used to build a scaffold around the granule upon initial fusion. Myosin II motors are subsequently recruited to the scaffold independently from F-actin. An Arp2/3 nucleated branched actin filament network emanates from the scaffold and drives rapid remodelling of the granule membrane. Inhibition of Arp2/3 nucleation eliminates the generation of the branched network. Subsequent contraction of the actin filament scaffold displays much slower kinetics to achieve identical remodelling of the granule membrane. This emphasizes the collaboration of multiple actin filament populations in the process of membrane remodelling during regulated granule exocytosis. At the basolateral membrane iSiMM was used to reveal single molecule dynamics of Tpm3.1 and myosin II motors. Molecular recruitment and nanostructures were analysed before and during exocytosis. A decrease in the diffusion coefficient of Tpm3.1 after stimulation indicate that remodelling at both the basolateral and apical membranes take place during exocytosis.

P989/B111

The Role of Non-muscle Myosin Motors in Actively Organizing Molecules at the Plasma Membrane.

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The plasma membrane of living cells is an active composite of a bilayer of lipids and proteins tightly coupled to an actin-based cytoskeleton. This configuration supports the nano and meso-scale organization of GPI-anchored proteins (GPI-AP) and Trans-membrane Actin binding proteins (TmABD). Theoretical and experimental work from our laboratory over the past decade[1-3] has argued for a central role for myosin motor activity in driving actin filaments into contractile aster-like configurations juxtaposed to the plasma membrane, which in turn can spatially pattern membrane molecules. However in non-muscle cells, greater than fifteen sub-types of myosins perform specialized functions and differ in terms of mechanical properties, localization and collective behavior. While it is known that the activity of various classes of non-muscle myosin motors impart contractility to the cytoskeleton, their precise role in the organization of membrane components is poorly understood. We employed genetic and pharmacological perturbation approaches to down-regulate the functions of different classes of non-

muscle myosins in mammalian cells. Using live cell FRET-based microscopy to obtain nano-scale information about the distribution of membrane molecules, we identify that distinct non-muscle myosin classes I and II are selectively required for the nanoscale organization of the different classes of membrane molecules, lipid-anchored (GPI-AP) and trans-membrane proteins with actin binding domains (TmABD). Additionally we observe that the lipid anchored GPI-AP and trans-membrane TmABD are organized into segregated domains at both nano (<10nm) and meso-scale (100nm-1μm) at the membrane. Our findings suggest that by independent regulation via the different non-muscle myosins, cells actively organize the lateral heterogeneity of the plasma membrane across multiple length scales. References 1. Gowrishankar, K. et al. Active remodeling of cortical actin regulates spatiotemporal organization of cell surface molecules. *Cell* 149, 1353-67 (2012). 2.Koster, Vasco, D. et al. Actomyosin dynamics drive local membrane component organization in an in vitro active composite layer. *Proc. Natl. Acad. Sci.* USA 113, E1645-E1654 (2016). 3.Goswami, D. et al. Nanoclusters of GPI-anchored proteins are formed by cortical actin-driven activity. *Cell* 135, 1085-97 (2008).

P990/B112

Membrane Binding of Actin Bundles Inside Giant Liposomes Promotes Condensation into Single Ring Structures.

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In cells, actin filaments are often organized into cross-linked, branched, or bundled networks. These different architectures appear in structures such as filopodia, stress fibers, the cell cortex, and contractile actomyosin rings; each has unique physical properties and fulfills different roles in important cellular processes. Bottom-up reconstruction of these fundamental eukaryotic features, and their stabilizing, as well as transformative functions on the cell membrane, will not only help us understand physiological processes, but is also an important stepping stone towards the engineering of artificial cells. In recent years, in *vitro* reconstitution of actin systems on synthetic vesicles and supported lipid bilayers has attracted great interest from physical biologists. However, encapsulation of actin inside cell-sized vesicles remains technically challenging. Here, we have adopted recent advances in vesicle encapsulation methods in order to study bundled actin filaments in giant unilamellar vesicles. We were thus able to compare actin organization mediated by different actin cross-linking proteins in a confined compartment, and directly visualize the effect of linking actin structures to a phospholipid bilayer. By changing a few key parameters, the assembly of actin bundles can be differentiated to resemble various types of actin networks in living cells in both structure and function. Importantly, we find membrane binding to be crucial for the robust condensation into single actin rings in vesicles. Once formed, the physical characteristics of the different actin network topologies can be directly observed. For example, cortices from bundled actin can act as a mechanically supportive scaffold that determines vesicle shape. With the application of force via myosin motors, ring-like actin bundles contract and can constrict the vesicle membrane inwards, a behavior that has not been reconstructed in *vitro* thus far. These observations confirm not only the suitability of this system for reconstituting processes such as cell division or migration, but also indicate the key role that membrane connections likely play in determining actin architecture and function in cells.

P991/B113

Sub-organellar Actin Dynamics Revealed by Organelle-targeted Actin Chromobodies.

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The actin cytoskeleton has long been known to regulate organelle dynamics. However, the small and transient actin structures regulating organelle dynamics are difficult to detect with fluorescence or electron microscopy. Live cell fluorescence imaging of organelle-associated actin is particularly hindered by the huge amounts of “background” signal caused by an unfortunate combination of (i) the ubiquity of actin throughout the cytoplasm, (ii) the relatively large and stable actin structures in the cell cortex of adherent cells, and (iii) the resolution limit optical microscopy. To circumvent this issue, we developed an approach using fluorescent protein-tagged actin nanobodies fused to organelle membrane targeting sequences. We validated the probes using pan-actin probes including phalloidin and cytoplasmic actin nanobodies. Fluorescence recovery after photobleaching measurements showed that these probes are highly diffuse on the organelle membrane unless bound to actin, which immobilizes them. Live cell imaging of these probes revealed previously undetected sub-organellar actin structures on mitochondria, the ER, and the nucleus. Simultaneous imaging of both ER- and mitochondria-associated revealed that actin first accumulates on mitochondria prior to the ER during mitochondrial fission events. Finally, we found ER-associated actin marks constriction or fission sites for all the organelles we tested, including mitochondria, endosomes, lysosomes, peroxisomes, and the Golgi. Overall, these results show that using organelle-targeted nanobody probes for studying organelle-cytoskeleton dynamics can reveal previously undetected structures in the cell with very high spatiotemporal resolution.

Kinesins

P992/B115

Mel-28-mediated Regulation of Microtubule Motors Affects Fertility in the Nematode *Caenorhabditis Elegans*.

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Microtubule motors are multi-molecular machines that ferry cargoes from one location to the other within a cell. We have been studying MEL-28, a conserved and essential protein important for chromosome segregation and the post-mitotic rebuilding of the nuclear pore. Previous work showed that MEL-28 and the minus-end directed microtubule motor dynein work in parallel to support fertility in *C. elegans*. Simultaneous disruption of *mel-28 and dhc-1* (which encodes the large subunit of dynein) causes low brood size and disorganization of the oogenic germline. Our main goal has been to understand why *dhc-1;mel-28* double mutants have reduced fertility. Using markers that identify oocyte stage, we have observed that *dhc-1;mel-28* double mutants have oocyte maturity defects. To find cellular components that regulate MEL-28 and dynein-related cellular processes, we did a candidate RNAi screen searching for genes that when disrupted rescue the brood size of *dhc-1;mel-28* double mutants. We found that disrupting *klc-2,*which encodes the light chain of the plus-end directed microtubule motor kinesin, drastically improves the brood size of *dhc-1;mel-28* double mutants. This suggests that MEL-28 and dynein work together to promote a cellular process that is opposed by KLC-2-

mediated kinesin activity. We are currently using RNAi to test known cargoes regulated by KLC-2 in order to determine if these are implicated in the rescue of *dhc-1;mel-28* fertility defects. All of the genes we are studying are conserved and essential in all animals. What we learn by studying genetic networks regulating fertility in *C. elegans* could be valuable for understanding regulation of intracellular trafficking in other animals, including humans.

P993/B116

A Means to an End: How a Non-Motile Kinesin Finds the Cilia Tip.

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The correct localization of Hedgehog effectors to the tips of primary cilia is critical for proper signal transduction. The conserved non-motile kinesin Kif7 defines a “cilium-tip compartment” by localizing to the distal ends of axonemal microtubules. How Kif7 recognizes microtubule ends remains unknown. We find that Kif7 preferentially binds GTP-tubulin at microtubule ends over GDP-tubulin in the mature microtubule lattice, and ATP hydrolysis by Kif7 enhances this discrimination. Cryo-electron microscopy structures suggest that a rotated microtubule footprint and conformational changes in the ATP-binding pocket underlie Kif7’s atypical microtubule-binding properties. Finally, Kif7 not only recognizes but also stabilizes a GTP-form of tubulin to promote its own microtubule-end localization. Thus, unlike the characteristic microtubule-regulated ATPase activity of kinesins, Kif7 modulates the tubulin mechanochemical cycle. We propose that the ubiquitous kinesin fold has been repurposed in Kif7 to facilitate organization of a spatially restricted platform for localization of Hedgehog effectors at the cilium tip.

P994/B117

Does Giraffe Kinesin Move Faster Than Mouse?

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Axonal transport has been demonstrated to be essential for various neuronal functions including neurite formation and extension, synaptic functions and survival. Many neurodegenerative diseases are known to be caused by the mutations in the genes related to this transport system. For example, various point mutations in the motor domain of KIF5A, a vertebrate specific neuronal isoform of kinesin-1, is known to be causative for an autosomal dominant form (SCG10) of hereditary spastic paraplegia (HSP), which mainly affects the distal part of the long motor tracts in the spinal cord. Most of the HSP mutations partially impairs the motor activity of KIF5A. Its velocity is reduced by 25-75% of the wild type. Considering that the mutated proteins are expressed at the similar amount to the wild type proteins, the transport would be only slightly slower in the heterozygous patients’ neurons. That would explain why neurons with longest axons are affected. If fast velocity is important for the survival of neurons with long axons, large animals with longer axons than human would require faster kinesin. Here, we addressed a simple question. Does kinesin of giraffe move faster than that of small animals such as mice? We have identified kinesin genes in the draft genome sequence of giraffe, and expressed recombinant giraffe KIF5A (GcKIF5A) protein. The motility of GcKIF5A was compared with mouse KIF5A (MmKIF5A) both in vitro and in cellulo. GcKIF5A moved about 25 % faster than MmKIF5A on purified neuronal microtubules in vitro and in axon, but similar velocity with MmKIF5A in dendrites and non-neuronal cells. Furthermore, transport of vesicles by GcKIF5A moved about 25 % faster than by

MmKIF5A in mouse hippocampus neurons. Interestingly, specific amino acid substitutions found in the motor domain of GcKIF5a were conserved only on burmese python KIF5a (PbKIF5a) among 150 species analyzed, and PbKIF5a moved significantly faster than MmKIF5a on purified neuronal microtubules in vitro. These results suggest that KIF5a of large animals with longer axons might have adapted for the longer axonal transport.

P995/B118

Cargo Diffusion Shortens Single-kinesin Runs.

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Molecular motors such as kinesin-1 drive active, long-range transport of cargos along microtubules in cells. Thermal diffusion of the cargo can impose a randomly directed, fluctuating mechanical load on the motor carrying the cargo. Recent experiments highlighted a strong asymmetry in the sensitivity of single-kinesin run length to load direction, raising the intriguing possibility that cargo diffusion may non-trivially influence motor run length. To test this possibility, here we employed Monte Carlo-based simulations to evaluate the transport of cargo by a single kinesin. Our simulations included physiologically relevant viscous drag on the cargo and interrogated a large parameter space of cytoplasmic viscosities, cargo sizes, and motor velocities that captures their respective ranges in living cells. We found that cargo diffusion significantly shortens single-kinesin runs. This diffusion-based shortening is countered by viscous drag, leading to an unexpected, non-monotonic variation in run length as viscous drag increases. To our knowledge, this is the first identification of a significant effect of cargo diffusion on motor-based transport. Our study highlights the importance of cargo diffusion and load-detachment kinetics on single-motor functions under physiologically relevant conditions.

P996/B119

Kinesin-based Transport Is Controlled by Cholesterol in the Cargo Membrane.

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Motor protein-based transport in cells underlies all eukaryotic cell function and survival; dysfunctions in this transport are implicated in many diseases, including neurodegeneration. While the properties of motor proteins have been extensively studied both in vivo and in vitro, many important questions remain, including how the properties of the cargo itself impact motor function. In cells, cargos are often membrane-bound; the composition of the cargo membrane has long been hypothesized to impact motor protein-based transport. However, quantitative investigation of this hypothesis is limited; most cargos in current in vitro assays lack a physiological membrane. To address this technical challenge, here we combined advances in membrane biophysics with established single-molecule optical-trapping experiments to characterize the transport of membrane-enclosed cargos in vitro. Our study focused on the major microtubule-based motor kinesin-1 and employed the microtubule-associated protein tau to mimic the crowded surface of the cytoskeletal tracks in cells. In contrast to the traditional, membrane-free approach, we found that coupling motors via a biomimetic membrane significantly enhanced the transport of cargos along tau-decorated microtubules. This effect diminished when we added cholesterol to our model membrane. Stochastic simulations indicated that the observed membrane effects reflect a change in the number of motors available to drive cargo transport. To our knowledge,

our study uncovers the first direct link between cargo-membrane composition and kinesin function. The experimental approach employed here is generally applicable as a controlled experimental platform for interrogating the control of motor proteins in a context directly relevant to in vivo scenarios.

P997/B120

Dexamethasone Accelerates Muscle Regeneration by Modulating Kinesin-1-mediated Focal Adhesion Signals.

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Skeletal muscle differentiation requires the fusion of mononucleated myoblast to form multinucleated muscle fibers. The glucocorticoid dexamethasone (Dex) is therapeutically applied to muscular dystrophy by inhibiting inflammation, slowing muscle degeneration and stabilizing muscle strength; nevertheless, how Dex promotes myogenesis remains unclear. Here we show that Dex directly accelerates kinesin-1 motor activity, which is required for the expression of muscle myosin heavy chain 1/2, the process of myoblast fusion and the formation of polarized myotube. Upon differentiation, kinesin-1 mediates the recruitment of integrin β1 onto microtubules for its delivery into focal adhesions. Integrin β1-mediated focal adhesion signals guide myoblast fusion in polarized morphology. By imposing geometric constrains via micropatterns, we have proved that cell adhesion is able to rescue the defects caused by kinesin-1 inhibition in the process of myogenesis. In adult and aged mice, we found that Dex significantly promotes muscle regeneration after injury through kinesin-1. These discoveries provide a mechanism of Dex in promoting myogenesis and lead us towards an efficient approach to improve the capacity for skeletal muscle regeneration with age.

P998/B121

Glycogen Synthase Kinase 3beta and Presenilin Mediated Regulatory Mechanisms of the Kinesin-1 Motor during Long Distance Axonal Transport.

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Long distance transport of essential components within axons is required for neuronal function and viability, and transport defects have been implicated as an early event in many neurodegenerative diseases including Alzheimer's disease (AD). Axonal transport is aided by kinesin-1 (anterograde) and dynein (retrograde) motor proteins on microtubules (MTs). One possible mechanism by which transport defects could occur is by improper regulation of these motors. Previously, we found that excess Glycogen synthase kinase 3β (GSK3β) cause transport defects by increasing the binding of motors to cargo. Presenilin (PS), a protein implicated in AD, also influences motor function. We identified that functional PS and the catalytic loop region of PS is essential for the rescue of GSK3β-mediated transport defects. Further, active GSK3β associated with and phosphorylated kinesin-1. Together, our observations propose a scaffolding mechanism for PS where the loop region sequesters GSK3β away from motors for proper regulation of motor function. To further identify the sites on kinesin-1 that GSK3β phosphorylates, we mutated three putative serine residues to generate phospho-defective and phospho-mimicking forms of kinesin-1 and found that only one of these sites is phosphorylated by GSK3β. Interestingly, only the phospho-mimicking kinesin-1 mutation abolished both kinesin-1 binding to MTs and MT motility, while the phospho-defective kinesin-1 mutation did not influence MT binding, but it impaired MT motility. Therefore, our findings identify the functional significance of PS-GSK3β-

mediated regulatory events on kinesin-1 motors, and isolate the mechanistic details of how GSK3β-mediated phosphorylation controls kinesin-1 activity during long distance axonal transport.

P999/B122

The Spatiotemporal Construction of the Axon Initial Segment Via KIF3/KAP3/TRIM46 Transport Under MARK2 Signaling.

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The axon initial segment (AIS) is a fundamental compartment serving as a molecular barrier to achieve axon-dendrite differentiation. Distribution of specific proteins within the early stage of neuronal development has been proposed to be critical for AIS construction. However, it remains unknown how these proteins are specifically targeted to the proximal axon within the limited time period. Here, we reveal spatiotemporal regulation driven by the microtubule (MT)-based motor KIF3A/B/KAP3 that transports TRIM46 under the specific MARK2 phosphorylation cascade. In the proximal part of the future axon under low MARK2 activity, the KIF3/KAP3 motor recognizes TRIM46 as cargo and transports it to the future AIS. In contrast, in the somatodendritic area under high MARK2 activity, KAP3 phosphorylated at serine 60 by MARK2 cannot bind with TRIM46 and transport. This spatiotemporal regulation between KIF3/KAP3 and TRIM46 under specific MARK2 activity underlies the specific transport for axon differentiation.

P1000/B123

Direct Competition between Molecular Motors Defines Posterior Determination in *Drosophila* Oocytes.

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The posterior pole of the *Drosophila* embryo is defined by an accumulation of *oskar (osk*) mRNA in the oocyte. Defects in *osk* mRNA localization prevent germ cell formation and abdomen specification. It is known that two major motor proteins, kinesin-1 and myosin-V, are essential for *osk* mRNA posterior localization. In this study, we used Staufen, an RNA-binding protein that colocalizes with *osk* mRNA, to visualize posterior determination in the oocyte. We find that the posterior localization of Staufen depends on three activities of kinesin-1: directed transport of Staufen particles toward the posterior pole, sliding microtubules to drive cytoplasmic streaming, and inhibition of Staufen particle accumulation at sites other than the posterior pole. Posterior localization of Staufen is defined by competition between kinesin-1 that removes Staufen from the cortex and myosin-V that anchors it. The outcome of this competition is determined by cortical microtubule density: it is won by myosin-V at the posterior pole where the microtubule density is low but not at the other parts of the oocyte cortex. In agreement with this model, local depolymerization of cortical microtubules in the lateral cortex using optogenetics causes ectopic accumulation of Staufen. Thus, direct competition between two motors defines the initial localization of posterior determinants in the *Drosophila* oocyte.

P1001/B124

Kif3b Transports Nr2a Complex and Supports Neuronal Plasticity and Functional Synapse Formation.

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Dysfunction of KIF3B, a member of the kinesin superfamily proteins (KIFs), causes schizophrenia (SCZ)-like phenotypes in *vivo* and in *vitro*. Here, we show that KIF3B supports the transport of vesicles simultaneously containing *N*-methyl-D-aspartate receptor (NMDAR) subunit 2A (NR2A), but not NR2B, and the adenomatous polyposis coli (APC) complex. *Kif3b*$^{+/-}$ neurons exhibited a reduction of synaptic levels of both NR2A and NR2B due to the impaired transport of NR2A and increased degradation of NR2B. The transport of NMDARs is crucial for neuronal plasticity and functional synapse formation. Indeed, electrophysiological recordings revealed that the reduction in the surface NMDAR expression of *Kif3b*$^{+/-}$ neurons altered their synaptic plasticity, which is a common basis of SCZ. A mutation of KIF3B was specifically identified in human SCZ patients and the SCZ-specific mutation-carrying KIF3B, but not wild-type KIF3B, failed to reverse the *Kif3b*$^{+/-}$ phenotypes in *vitro*. Therefore, we propose that SCZ-like phenotypes in KIF3B deficiency is caused by the defects in the transport and function of the NR2A/APC complex.

P1002/B125

Allosteric Effects of Mutations in the Kif1a Motor Domain Associated with Neurodevelopmental Disorders.

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The functional output of the microtubule (MT) cytoskeleton depends on a family of molecular motor proteins called kinesins. For transport kinesins, the catalytic motor domain converts the chemical energy of ATP hydrolysis into the mechanical outputs of force generation and directed “stepping” along MT tracks. KIF1A, a member of the kinesin-3 family, is a fast, super processive motor that is critical for the transport of presynaptic vesicles in neurons. Mutations in the KIF1A motor domain have been linked to neurodevelopmental disorders, however we have a limited understanding of how these mutations impact the functional output of the motor domain. Here, we used an interdisciplinary approach to determine the functional impact of two KIF1A mutations located at or near positions involved in kinesin force generation: V8M in beta-1 and Y89D in alpha-1/beta-3. Molecular dynamics (MD) simulations predict that Y89D impairs neck linker docking but not cover-neck bundle formation, suggesting that this mutation decreases force generation of the motor. Both mutations are predicted to have allosteric effects on (1) ATP binding/hydrolysis, and (2) association with the MT track. We tested these predications using single-molecule motility and optical-trapping assays. We find that the mutant motors are slower, less processive, and have a higher frequency of nonproductive, diffusive events along the MT. These motility properties are only modestly rescued when a mutant motor domain is paired with a wild-type motor domain in a heterodimeric motor. Furthermore, Y89D dramatically impairs the motor’s ability to generate force and remain attached to the MT track when under load. Our results highlight

how single residue changes in the motor domain can have allosteric effects that alter the functional output of the motor. This work provides insight into the structural elements critical for kinesin-3 motility, and advances our understanding of how mutations in the kinesin motor domain can manifest in disease.

P1003/B126

Using Protein Interaction Networks to Understand Kinesin-based Hereditary Spastic Paraplegia.

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Hereditary Spastic Paraplegia (HSP) is a progressive neurodegenerative disease that causes spasticity and weakness in the lower limbs due to denervation of neurons in the corticospinal tract. To date, HSP has been shown to be caused by a variety of mutations in 74 separate human genes (SPGs), as well as another 13 distinct genetic loci. While subsets of the SPGs share gene ontology groupings or participate in broad cellular processes, there is no clear biological pathway that is common among the separate genes that could tie them to a single cellular disease mechanism. The bulk of the existing HSP research has focused on individual SPGs and the altered function of proteins that result from HSP-causing mutations. While this has shed light on the functional deficits associated with mutation in each gene, it has not led to insight in the shared cellular process that is abrogated in the context of HSP. We have taken a different approach by attempting to identify potential interactions between the protein products of SPGs to link them to a common cellular function. To do this, we began with a bioinformatics approach to glean data from existing protein-protein interaction databases. We downloaded the BioGRID database in TAB 2.0 format and built an undirected graph in PERL. We identified SPGs as vertices in the graph, then performed a breadth first search to identify direct interactions (vertices that were neighbors) or indirect interactions (vertices that were neighbors of neighbors). While there were a number of single interactions between SPGs, three larger nodes were identified, the largest of which was centered on the neuronally enriched conventional kinesin, KIF5A. This node contained KIF5A (SPG10), two kinesin light chains (KLC2/SPG68 and KLC4/SPG), and three additional SPGs (SPG20, SPG13, and SPG74). When we expanded the analysis to indirect interactions between SPGs, the KIF5A-containing node expanded to include 13 additional SPGs that indirectly interact with KIF5A. In recognition that this approach is only as complete as the starting data set, we attempted to develop a more complete set of KIF5A-interacting proteins. We first performed a 2-hybrid screen using the cargo binding domain of KIF5A as bait, and screening a human brain cDNA library. This identified 90 distinct genes whose protein products could interact with KIF5A. We are in the process of carrying out a secondary screen using the proximity biotinylation assay to distill the data such that we can follow up on the overlapping data sets from each screen.

P1004/B127

Cargo-mediated Activation of KIF1C Transport.

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The kinesin-3 KIF1C is a fast organelle transporter implicated in the transport of dense core vesicles in neurons and the delivery of integrins to cell adhesions. Mutations in KIF1C result in hereditary spastic paraplegia (HSP) and cerebellar dysfunction. Here we show that the activity of KIF1C is controlled by its

own stalk domain, which competes with the microtubule binding interface of the motor domain. These autoinhibitory, intermolecular interactions are released when the cargo adapter HOOK3 or the protein tyrosine phosphatase PTPN21 bind to the stalk domain. Both activators increase the landing rate of the motor and remain bound to KIF1C during transport. The FERM domain of PTPN21 stimulates dense core vesicle transport in primary hippocampal neurons and rescues integrin trafficking in KIF1C-depleted cells, while HOOK3 mediates the formation of a bidirectional transport complex of KIF1C and dynein/dynactin. In vitro, full length human KIF1C is a highly processive, plus-end directed motor with a stall force of 5.5 pN. Removing the stalk domain results in a hyperactive motor with a 20-fold increased landing rate and 5-fold increased speed that is, however, unable to functionally replace KIF1C in cells. On the other hand, mutations in the KIF1C motor domain found in HSP patients, result in slower and weaker motors that are still processive, but cannot generate forces of more than 2 pN. Our findings reveal a mechanism to control the activity of the major neuronal transporter KIF1C and a molecular understanding of the altered mechanical properties of the motor resulting from human disease mutations.

P1005/B128

Analysis of the Immotile Kinesin-4 Motor Kif7 and Its Role in Hedgehog Signaling.

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Hedgehog signaling plays critical roles during development and tissue homeostasis. The kinesin-4 motor KIF7 is a conserved regulator of Hedgehog signaling and mutations in KIF7 are linked to human ciliopathies. In response to Hedgehog pathway activation, KIF7 localizes to the tip of the primary cilium in mammalian cells and facilitates the localization of Hedgehog effectors to the same location. However, unlike conventional kinesins, KIF7 is immotile and its ability to interact with microtubules is not regulated by ATP binding or hydrolysis. How the microtubule binding of KIF7 is regulated and relates to its functions in Hedgehog signaling are unclear. We demonstrate that KIF7 is regulated by autoinhibition as full-length KIF7 does not bind to microtubules whereas truncated motors bind statically to all microtubules in cells, including along the ciliary axoneme. Thus, unlike the behavior of purified motors in *in vitro* assays, truncated versions of KIF7 do not bind selectively to or track the plus ends of microtubules in cells. Using an inhibitable version of the heterotrimeric kinesin-2 motor KIF3A/KIF3B/KAP, we demonstrate that kinesin-2-driven intraflagellar transport (IFT) is required for localization of KIF7 to the cilium tip in response to Hedgehog stimulation. To understand how the unique microtubule-binding features of KIF7 are adapted for its role in Hedgehog signaling, we generated KIF7 variants with altered microtubule interaction phenotypes (no microtubule binding, increased microtubule binding, or microtubule-based motility) and examined their localization and functional output in KIF7-/- MEF cells. We demonstrate that KIF7 motors that lack microtubule binding activity behave similar to wild-type motors in their ability to a) localize to the cilium tip and b) facilitate accumulation of the Gli2 and Gli3 transcription factors at the cilium tip in response to Hedgehog pathway activation. In contrast, KIF7 motors capable of microtubule-based motility can a) localize to the cilium tip and b) drive the localization of Gli2 and Gli3 to the cilium tip even in the absence of Hedgehog pathway activation. Together this work suggests that autoinhibition and the immotile behavior of KIF7 are important for preventing the localization of Hedgehog effectors to the primary cilium in absence of Hedgehog signaling.

P1006/B129

Motor-specific Regulation by Maps - Tau and Map7 Differentially Regulate Kinesin and Dynein Motors to Direct Transport of Intracellular Cargoes.

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Cargoes are shuttled to different locations in the cell along microtubules by plus-end directed kinesin and minus-end directed dynein motors. While many cargoes are transported by similar sets of kinesin and dynein motors, they exhibit different transport characteristics and are localized differently in the cell. Emerging evidence suggests that microtubule associated proteins (MAPs) regulate kinesin and dynein to target intracellular transport. Two neuronal MAPs, tau and MAP7, control the motility of organelles by regulating the forces exerted by kinesin and dynein teams. We isolated endogenous cargoes driven by teams of kinesin-1, kinesin-2, and dynein motors and reconstituted their motility in vitro. On tau-decorated microtubules, we observe more minus-end directed motility, while cargoes moving along MAP7 decorated microtubules demonstrate more plus-ended motility. Furthermore, the specific set of motor proteins associated with a cargo determine how its transport will be affected by MAPs. Early endosomes are enriched for kinesin-1, while kinesin-2 is the dominant plus-end directed motor on late endosomes. Previous studies have shown that tau inhibits kinesin-1 more strongly than kinesin-2 or dynein. Accordingly, we observe that early endosomes are more sensitive to tau expression in COS-7 cells. Collectively, our results indicate that MAPs can differentially regulate the load-dependent binding and unbinding rates of specific motor proteins to enable cargo trafficking to precise locations in the cell.

P1007/B130

Mitochondria-adaptor TRAK Enables Kinesin-1 Driven Transport in Crowded Environments.

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Intracellular trafficking of organelles driven by kinesin-1 stepping along microtubules underpins essential processes, such as neuronal activity. Numerous experiments in the absence of other proteins show that during a single microtubule attachment kinesin-1 performs hundreds of steps resulting in micron-long runs along the microtubule. The presence of other microtubule-associated proteins, however, decreases drastically the lengths of kinesin-1 runs. How can kinesin-1 act as an efficient transporter in the crowded cellular environment thus remains largely unclear. Here, we in *vitro* show that the interaction of TRAK (Milton), an adaptor protein essential for mitochondrial trafficking, activates kinesin-1 and enables kinesin-1-based transport in crowded environments. The binding of TRAK to kinesin-1 i) increased the probability of kinesin-1 passing through cohesive envelopes of tau molecules formed on the microtubule surface, ii) facilitated kinesin-1 navigation around obstacles on microtubules and iii) increased the run length of kinesin-1 in cell lysate. Moreover, TRAK enabled kinesin-1-based long-distance transport of isolated mitochondria along microtubules in vitro. We explain this enhanced transport activity of kinesin-1 by an observed interaction of TRAK with microtubules, which provides an additional foothold for the stepping of the TRAK-kinesin-1 complex. We show that anchoring of kinesin-1 to the microtubule

by an adaptor protein can turn kinesin-1 into an efficient long-range transporter in crowded environments.

P1008/B131

Kinesin-associated Protein KAP3 Is a Neuronal Vesicle Adaptor for KIF3AB and KIF3AC.

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Cells require precise localization of proteins for proper health and function. This is especially evident in neurons, where elongated processes contain distinct protein populations which must be transported long distances from the soma to their appropriate locations. Proteins are packaged into vesicles that are transported by molecular motors such as kinesins or dynein. These motors typically use adaptor proteins to facilitate binding to their vesicles. The Kinesin-2 family members KIF3AB and KIF3AC uniquely differ from conventional kinesins in that they are heterodimers instead of homodimers. KIF3AB is the major anterograde transporter for IFT particles, using KAP3 as an adaptor protein. Much less is known about KIF3AC. Both kinesins are expressed in neurons, but their neuronal functions are unknown. Here we show that KAP3 is a neuronal vesicle adaptor for both KIF3AB and KIF3AC. In cultured hippocampal neurons, KIF3AB and KIF3AC each bind organelles that have similar distributions. They are localized to soma and dendrites and excluded from axons.The identity of these organelles is not known. To determine whether KIF3AB and KIF3AC reside on the same organelles, we co-expressed fluorescently tagged KIF3B or KIF3C in cultured hippocampal neurons, each along with unlabeled KIF3A to ensure dimerization. We found substantial overlap between KIF3B and KIF3C, indicating that KIF3AB and KIF3AC can bind the same organelle. Additional two-color experiments found that KAP3 co-labeled vesicles with KIF3B and KIF3C. This result supports the hypothesis that KAP3 acts as the adaptor for both KIF3AB and KIF3AC. To test this directly, we utilized the well-characterized FRB-FKBP heterodimerization system to artificially target KAP3 to peroxisomes in cultured hippocampal neurons. Fluorescently labeled KIF3B and KIF3C do not normally localize to peroxisomes. Targeting of KAP3 to peroxisomes caused the recruitment of KIF3B and KIF3C to these organelles, while it did not recruit the Kinesin-1 family member KIF5C. This shows that KAP3 binds to Kinesin-2s KIF3AB and KIF3AC in neurons and supports the hypothesis that it acts as a neuronal vesicle adaptor for both heterodimeric kinesins. Together, these results show for the first time that KAP3 is a neuronal vesicle adaptor for KIF3AC and show that KAP3 interacts with KIF3AB.

P1009/B132

ARL8 Dependent Autoinhibition of SKIP Regulates Its Association with Kinesin-1.

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Intracellular transport and positioning of organelles rely on coupling to motor proteins that move bidirectionally along microtubule tracks. Coupling of organelles to motors is mediated by specific adaptor proteins. The correct spatio-temporal functions of the motors are crucial to maintain cellular processes. Indeed, malfunctions in this machinery are the cause of numerous pathologies including amyotrophic-lateral-sclerosis and hereditary-spastic-paraplegia. Here, we focused on lysosomes, which are highly dynamic organelles. Recent evidence indicates that the precise positioning of lysosomes in the cell is important for autophagy, nutrient signaling and plasma membrane repair. The outward movement of lysosomes is regulated by a lysosome-associated complex named BORC that initiates a chain of interactions involving the small GTPase ARL8, the adaptor protein SKIP, and kinesin-1. We used

bioinformatics, biochemical and cell biology tools to identify elements that regulate lysosome positioning, focusing on the adaptor protein SKIP. We found that dimerization of SKIP is required for its function. Most importantly, we discovered that SKIP is inhibited by an intra-molecular interaction between its N- and C-terminal parts. This inhibition is relieved by interaction with ARL8, indicating that ARL8 promotes conformational activation of SKIP for coupling of lysosomes to kinesin-1. More generally, these findings uncover a novel regulatory mechanism in which an organelle adaptor changes from an autoinhibited to an active conformation upon interaction with regulators on the organellar membrane.

P1010/B133

Ramp (Reversible Association with Motor Proteins): a Novel Method to Control the Positioning of Organelles within the Cell.

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Recent advances in live-cell imaging have revealed that the spatial organization of cytoplasmic organelles is highly dynamic. These organelles move back and forth between the center and the periphery of the cell along microtubule tracks. Organelle positioning and movement are critical for maintenance of cellular homeostasis, but the connection of these properties to organelle function remains unclear. This limitation is due to the lack of strategies to manipulate organelle movement and positioning. Therefore, we developed a new method named RAMP (for Reversible Association with Motor Proteins) to control the positioning of different organelles in the cytoplasm. RAMP consists of co-expressing in cells (i) an organellar protein fused to the streptavidin-binding peptide (SBP), and (ii) a microtubule motor fused to streptavidin. Binding of SBP to streptavidin forces the coupling of the organelle to the microtubule motor. The use of plus- or minus-end directed kinesin motors causes accumulation of organelles in the peripheral or perinuclear cytoplasm respectively. Addition of biotin disrupts the SBP-streptavidin interaction, allowing the return of the organelle to its normal steady-state distribution. Using this method, we could manipulate the positioning of various organelles in non-polarized cells: lysosomes, mitochondria, peroxisomes, and ER-resident proteins. This method has been also applied in hippocampal neurons in culture. In these polarized cells, RAMP can be used to accumulate cargo in the perinuclear area and study its trafficking into the dendrites and axon, but also to force the entry of cargo into the axon and study its retrograde transport back to the soma. When applying RAMP to mitochondria, we found that they became rounder and smaller when accumulated at the cell periphery. In contrast, accumulation of mitochondria at the cell center had no effect on their shape. These observations open the possibility of using RAMP to study the mechanisms involved in fission-fusion of mitochondria and their relationship with the pulling forces of motor molecules. We also used RAMP to manipulate the distribution of the ER by using an engineered ER-resident protein. The redistribution of the ER was partial and did not lead to complete disruption of the ER network. The residual structures could be resistant to redistribution because they are tightly tethered to the nucleus, the plasma membrane, or cytoskeletal structures. ER exit sites (ERES) also underwent redistribution after RAMP demonstrating that ER-associated structures can also be manipulated with this tool. In conclusion, RAMP is a new tool for manipulating organelle positioning, which, because of its distinct features, ease of use, and versatility may find broad application in the study of organelle biology.

P1011/B134

Development of Chemical Probes That Target the Mitotic Kinesin Kif15.

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Many anti-proliferative drugs used to treat cancer have off-target effects on non-dividing cells. To circumvent this shortcoming, drugs are now being developed to target proteins with roles specific to dividing cells. Promising targets include mitotic kinesins such as Eg5, which drives formation of the mitotic spindle; however, while effective in *vitro*, Eg5 inhibitors have failed clinical trials. One explanation for this discrepancy is that a second kinesin, Kif15, suppresses the cytotoxic effect of Eg5 inhibitors in *vivo* by driving bipolar spindle assembly independent of Eg5 activity. This led to the hypothesis that targeting both Eg5 and Kif15 is an effective strategy for undermining spindle assembly in dividing cells. A novel small molecule Kif15 inhibitor was identified by the Ohi lab during a screen of the GlaxoSmithKline library of Published Kinase Inhibitors. This compound, GW406108X, potently inhibits Kif15 and can effectively disrupt bipolar spindle assembly in Eg5-inhibitor-resistant cells, but has off-target effects due to its anti-kinase activity. To improve our chances of identifying a successful Kif15 inhibitor, I screened the MB 24K Maybridge library of small molecules at University of Michigan's Center for Chemical Genomics using an ATPase assay that correlates ATPase activity with luminescence. Primary screening and secondary hit follow-up of the MB 24K Maybridge library was completed, and the pool of compounds was narrowed down from 24,000 library compounds to 4 potential Kif15 inhibitors. Preliminary experiments suggest these compounds have minimal off-target affects and can block bipolar spindle assembly. I will present an update of my screen, strategies I used to confirm compounds that scored positively, and assays I employed to evaluate and prioritize hits. In conclusion, we have identified four potential Kif15 inhibitor scaffolds that potently and specifically block Kif15 activity both in *vitro* and in cells.

P1012/B135

Biochemical Characterization of the KIF1A Chemomechanical Cycle.

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Kinesin-3 is the fastest and most processive neuronal transport kinesin, yet important mechanistic features, such as the cause for binding of the tethered head and the identity of the rate-limiting step(s), are poorly understood. Superprocessivity requires a high probability that the motor will step rather than detach, so understanding the biochemical initiation of the forward step is paramount. We proposed three potential triggers for binding of the tethered-head to the next tubulin: 1) no trigger, 2) ATP-binding trigger, or 3) ATP-hydrolysis trigger. A trigger-less step would minimize the one-head-bound state (1HB), and an ATP binding trigger would maintain the motor in a tightly bound state, making each an attractive explanation for superprocessivity. In contrast, a hydrolysis trigger, which has been recently established for kinesin-1, involves a post-hydrolysis weakly-bound state susceptible to detachment, a feature that hinders superprocessivity. To test these models, we used stopped-flow fluorescence spectroscopy, steady-state kinetics, and single-molecule motility assays to characterize the chemomechanical cycle of the kinesin-3, KIF1A. The KIF1A on-rate, k_{on}^{Mt}, is 17.3 $\mu M^{-1}s^{-1}$, 20-fold faster than kinesin-1. The k_{cat} from solution ATPase assays is 278 s^{-1}, in agreement with the fast motility. KIF1A releases one ADP when combined with nucleotide-free microtubules, ruling out the no-trigger model. When this 1HB complex was combined with ATPγS, the tethered-head ADP release rate was 16.9 s^{-1}

whereas ATP triggered half-site release was 162 s^{-1}. This result indicates ATP hydrolysis occurs before tethered head binding and undermines the ATP-binding trigger model. The similarity of the half-site release rate to the k_{cat} suggests the rate-limiting step is before or during the release of ADP. We conclude that KIF1A exercises the same general stepping framework as other neuronal transport families but differs in the kinetics of specific transitions within the chemomechanical cycle.

P1013/B136

In Vitro Reconstitution of Kinesin-driven Vesicle Transport.

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Intracellular vesicle cargos are transported along microtubules by kinesin and dynein motors, towards the cell periphery and cell center, respectively. Individual motors generally have run lengths of several μm, whereas organelles need to travel tens of μm to reach their destinations. Therefore, vesicle cargos usually have multiple copies of motors such that when one or more motors detach from the microtubule, other motors can continue transport. We previously used a 2D lipid bilayer system to measure the microtubule binding kinetics of membrane-bound kinesin-1 motors, from which we predicted long-range transport of 100 nm diameter vesicles required 35 kinesin-1 motors. Our predicted motor number was higher than what was measured on purified neuronal vesicles, thus we aimed to reconstitute kinesin-driven vesicle transport and characterize vesicle motility behaviors in the present study. GFP-labeled kinesin-1 was linked to POPC liposomes through a DNA linker. As the mixing ratio of kinesin-1 and vesicle increased, the vesicle landing density in AMPPNP increased linearly and then reached a plateau. The average vesicle travel distance in ATP increased continuously with the mixing ratio, indicating that multi-motor transport was achieved. Vesicle transport velocity was insensitive to motor number. This system enables reconstitution of vesicle motility with controlled motor densities and can be extended to study bidirectional transport by kinesins and dyneins.

P1014/B137

Tracking Down the Fast and Superprocessive Kif1a with Gold Scattering Microscopy.

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The kinesin-3 family member KIF1A is a neuronal kinesin that performs long-distance anterograde vesicle transport in axons and dendrites. Single molecule studies observe KIF1A velocities > 1 μm/s and average run lengths > 5 μm, making KIF1A one of the fastest and most processive members of the kinesin superfamily; however, the mechanistic basis of these high speeds and long run lengths is unknown. One prevailing model for superprocessivity holds that the positively-charged “K-loop” in the KIF1A motor domain diffusively tethers the motor to the negatively-charged microtubule, which prevents complete dissociation of the motor and effectively links together short runs. However, this model does not account for how KIF1A reaches such high speeds, or what role the K-loop plays in the ATP-driven stepping mechanism. To address these questions, we labeled the C-terminus of a truncated KIF1A dimer with a 30-nm gold nanoparticle and used interferometric scattering microscopy (iSCAT) to observe the KIF1A stepping cycle at sub-millisecond time scales and nanometer-level precision. Our preliminary data reveal that KIF1A motility is characterized by periods of directed stepping interspersed by periods where the motor is statically bound to the microtubule, rather than diffusively tethered, as predicted by the K-loop model. An alternate explanation for superprocessivity, that could also account for the stepping speed, is that during each step the K-loop ensures the tethered head binds to the

microtubule hundreds of times faster than the bound head detaches from the microtubule. Our ongoing investigations employ a KIF1A construct with a nanoparticle-functionalized motor domain to characterize the nature of these paused states, and the duration of the one-head bound state in the chemomechanical cycle.

P1015/B138

Modulation of Kif18b Function through Regulatory Interactions of Its Tail.

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Tight regulation of microtubule dynamics and organization is necessary for proper spindle assembly and chromosome segregation. One important mitotic regulator is the Kinesin-8 motor Kif18B, which localizes to the plus-ends of astral microtubules and regulates their length and number during prometaphase. Using yeast two-hybrid and in *vitro* pull-down assays, we previously showed that Kif18B can interact with the plus-tip tracking protein EB1, as well as with the nuclear transport receptor importin alpha. Here, we want to understand how the binding of Kif18B to EB1 and importin alpha affects its function in mitotic spindle assembly. We developed in *vitro* FRET biosensors to examine the interactions of Kif18B with EB1 and importin alpha and to map their binding sites. Using these biosensors, we found that the tail of Kif18B interacts independently with either EB1 or importin alpha/beta and that binding of EB1 to Kif18B can be competitively inhibited by importin alpha/beta. The tail of Kif18B also binds to microtubules, and its binding to microtubules is inhibited by importin alpha/beta, but not by EB1. Using site-directed mutagenesis, we identified two EB1 Binding Domains (EBBD) and two Nuclear Localization Signals (NLS) in the tail of human Kif18B. Mutation of both EBBD sites was required to abolish interaction with EB1, but these mutations had no effect on microtubule binding. Similarly, mutation of both NLS sites was required to abolish intermolecular FRET between the tail of KIF18B and importin alpha. Mutation of the NLS sites also reduced the affinity of the tail for microtubules, suggesting that importin alpha binding is used to modulate the interaction of Kif18B with microtubules. To examine how the interaction of Kif18B with each of these modulators affects microtubule dynamics in mitosis, we are using lentiviral transgene delivery to express eGFP tagged Kif18B wild type and mutant constructs in cells in which the endogenous Kif18B has been knocked down. We have created a series of single and double mutant constructs for both NLS and EBBD binding sites that also have silent mutations that allow the siRNA to selectively knockdown the endogenous Kif18B, while not silencing the expressed transgenes. By examining the mitotic spindle morphology as well as measuring the size and density of the astral microtubule array, these studies will reveal how differential interactions of Kif18B with its binding partners modulate Kif18B activity in mitosis.

P1016/B139

Endogenous Kif25 Associates with Centrosomes to Oppose Centrosome Separation during S and G2 Phases of the Cell Cycle.

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Kif25 is a minus-end directed bipolar tetrameric kinesin which is expressed at low levels in cultured human cells. Depletion of endogenous Kif25 protein with siRNA elicited premature centrosome separation in S and G2 cells. Premature centrosome separation also led to stochastic mis-positioning of

the nucleus which destabilized spindle positioning when cells entered mitosis. Previously, we have shown by RT-PCR that Kif25 mRNA is transcribed in HeLa cells. We have prepared polyclonal antibodies against both the motor and the N-terminus of Kif25. Using an ATP-free microtubule pelleting assay to enrich for kinesins we have identified full-length Kif25 as a protein that co-purifies with microtubules in an ATP-dependent manner from both HeLa and Hct116 human cell lines. Affinity purified antibodies raised against expressed Kif25 motor cross-reacted with a 64kD protein consistent with the predicted size of full-length Kif25. Addition of ATP to the microtubule pellet releases Kif25 into the supernatant consistent with its identity as an ATP-dependent motor. Mass spec analysis indicates that Kif25 interacts with CEP170, a centrosomal protein that has also been shown to target kinesin-13 family members to the centrosome. We have prepared a Neon-green (NG)-FKBP-Kif25 transgenic CRISPR human cell line which enables us to visualize Kif25 expressed at endogenous levels. Endogenous NG-FKBP-Kif25 colocalized with pericentrin and with centrosomal satellites. The localization is dynamic in live cells. Depletion of protein with siRNA directed against Kif25 eliminated the endogenous NG-FKBP-Kif25 fluorescence. The addition of the FKBP moiety to the first intron of Kif25 allowed us to relocalize the protein from centrosomes to FRB-BFP anchored in the plasma membrane by the addition of Rapamycin. Relocation of exogenous Kif25 to the membrane led to premature centrosome separation in S and G2 cells, similar to what we have seen using siRNA depletion of Kif25. Kif25 protein is expressed at low levels in all tissues and, as a result, is often overlooked in screens. This is why detection of Kif25 full-length protein requires enrichment on microtubules in the absence of ATP. Some studies have reported higher median Kif25 expression in brain, adipose, testis and kidney tissue than in other tissues (GTEx an alysis Release V8). We investigated Kif25 expression in human NTera-2 cells which differentiate along a neural pathway when serum is withdrawn. Interestingly, we observed more centrosomes with associated Kif25 label in differentiating neurons as compared to undifferentiated cells in serum suggesting that Kif25 facilitates centrosome cohesion during neuronal differentiation as well as during S and G2 in other cultured human cell lines.

P1017/B140

Mutations in the Chromokinesin KIF22 Disrupt Mitotic Chromosome Segregation and Cause Skeletal Dysplasia.

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The chromokinesin KIF22 (Kid) generates polar ejection forces and contributes to anaphase chromosome compaction in mitotic cells. The structure of this protein includes a conserved kinesin motor domain, a second microtubule binding domain in the tail, a coiled-coil domain, and a C-terminal DNA binding domain. Mutations in *KIF22* dominantly cause spondyloepimetaphyseal dysplasia with joint laxity- leptodactylic type (SEMDJL2), a skeletal developmental disorder. Published exome sequence analyses of SEMDJL2 patient samples have identified mutations in proline 148 and arginine 149 of the motor domain α2 helix as causative of disease pathology. Additionally, we report that two new skeletal dysplasia patients have point mutations in the coiled-coil rather than motor domain of KIF22. To assess the effect of SEMDJL2 patient-derived mutations on the mitotic function of KIF22, we have built stable cell lines that inducibly express KIF22-GFP with SEMDJL2 causative mutations R149Q (motor domain) and V475G (coiled-coil domain). Expression of KIF22-GFP R149Q or V475G does not disrupt polar ejection force generation, and expression of either mutant motor rescues polar ejection force

generation in cells depleted of endogenous KIF22. We observe anaphase chromosome segregation defects and disrupted nuclear morphology following mitosis in cells expressing KIF22-GFP R149Q or V475G. These results suggest that SEMDJL2-causative mutations impair the anaphase, but not prometaphase, function of KIF22. We are currently investigating the mechanisms by which the effects of motor and coiled-coil domain mutations are specific to phases of mitosis, and why SEMDJL2 mutations result in a patient pathology specific to the skeletal system.

P1018/B141

Schizophrenia-like Phenotypes in Mice with Molecular Motorkif3bgenedeficiency.

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Schizophrenia (SCZ) is one of the most common neurodevelopmental disorders and is tightly linked to a variety of genetic factors. KIF3B is a member of the KIFs and forms a heterotrimeric complex (KIF3 complex) with KIF3A and kinesin superfamily-associated protein 3 (KAP3). Here we carried out a series of behavioral analyses and found that KIF3B dysfunction causes SCZ-like phenotypes. In the prepulse inhibition test (PPI), the extent of inhibition with the prepulse in Kif3b+/- mice was significantly smaller than that in Kif3b+/+ mice. Kif3b+/- mice exhibited a lower level of anxiety and social interest, whereas they exhibited a higher level of exploratory behavior and repetitive behavior such as jumping and rotating activity, and they spent more time grooming, suggesting locomotion hyperactivity. Kif3b+/- mice also had impaired spatial memory formation and learning flexibility, which is similar with other mouse models of SCZ. The histological features of Kif3b+/- mouse brain also mimicked SCZ features, which underlie behavioral defects of Kif3b+/- mice in PPI, the social interest, and learning flexibility. Therefore, we propose that KIF3B dysfunction causes SCZ-like phenotypes.

Microtubule Dynamics and its Regulation 2

P1019/B143

The Elongator Complex Controls Symmetry Breaking of the Central Spindle and Thereby Polarized Trafficking of Cell Fate Determinants during Asymmetric Cell Division.

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Asymmetric cell division gives rise to two daughter cells, which inherit different determinants, thereby acquiring different fates. The polarized sorting of signalling endosomes from an asymmetric central spindle has recently emerged as an important feature of asymmetric cell division in Drosophila Sensory Organ Precursors (SOP). However, how symmetry breaking of the central spindle occurs in vivo remains poorly understood. Here, we have identified a new player in this pathway, namely the Elongator

complex. Elongator is a 6-subunits multiprotein complex that has been involved in the regulation of transcription and translation through histone acetylation and tRNA methylation, respectively. We found that, in SOPs, Elongator localizes to the central spindle, and that deletion of the catalytical subunit Elp3 abolishes central spindle asymmetry and, accordingly, Sara endosome asymmetric segregation. As Sara endosomes contain the cell fate determinants Notch and Delta, this induces cell fate phenotypes in the adult fly. We further investigated the molecular mechanism by which Elongator breaks the symmetry of the central spindle. Mammalian Elongator has previously been proposed to acetylate microtubules, thereby potentially controlling their dynamics. However, we found that purified Drosophila Elongator does not acetylate tubulin in vitro and that Elp3 mutants can be rescued with an acetylase-dead version of Elp3 in vivo. Rather, we found that Elongator binds to dynamic microtubules and lowers the frequency of microtubule catastrophe in in vitro reconstituted single molecule assays. This data suggests that Elongator asymmetrically stabilizes microtubules on one side of the central spindle, thereby generating central spindle asymmetry. We demonstrate this by modifying the Elongator activity gradient through asymmetric targeting of Elongator to the anterior cortex, which accordingly perturbs Sara endosome asymmetric segregation. Altogether, this data suggests that Elongator constitutes a novel link between polarity cues at the cell cortex and symmetry breaking of the microtubule cytoskeleton in the cytosol.

P1020/B144

Investigating the Role of Lateral Interactions in Microtubule Dynamics.

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Microtubule dynamics are driven by tubulin subunits making longitudinal and lateral contacts with neighboring subunits in the microtubule polymer. While recent structural data suggests that polymerization involves structural changes at the longitudinal interface, we know less about the role lateral interactions play. **We hypothesize that the lateral interface determines how tubulin forms the cylindrical microtubule lattice and plays an important role in microtubule dynamics**. To test this hypothesis, we investigate how changes across the lateral interface affect microtubule polymerization and depolymerization, focusing on the M-loop of β-tubulin and its interaction with H1-S2 of the neighboring β-tubulin subunit. Based on previous EM studies, the lateral interactions are highly sensitive to temperature decrease. We first measured that effect of temperature on microtubule polymerization, depolymerization, and length. We found all parameters scale with temperature, with the depolymerization rate being more affected by changes in temperature than polymerization. To test the prediction that lateral interfaces determine the microtubule's response to temperature, we generated mutations in the yeast β-tubulin gene, *TUB2*, to mimic amino acid substitutions found in the H1-S2 and M loops of an tarctic species that exhibit cold stable microtubules. We find that an S278G mutation in the M loop increases microtubule dynamics and lowers the temperature dependence of microtubule dynamics by decreasing the activation energy for both polymerization and depolymerization. In contrast, a S56D mutation in the H1-S2 loop affects polymerization and depolymerization differently. We predict that the S56D substitution creates an additional charged-charged interaction across the lateral interface. To assess the relative contributions of lateral and longitudinal interactions during microtubule polymerization, we used combinations of the β-tubulin lateral loop substitutions with mutations known to disrupt the longitudinal interface. We propose a model for microtubule polymerization in which the longitudinal interface governs the on/off rate of a tubulin dimer at the protofilament level, and the lateral interface dictates how quickly protofilaments interact to create a

cylindrical lattice. This work extends our knowledge of how the less well understood lateral interface contributes to microtubule dynamics.

P1021/B145

Synthetic Effects of H^+, Na^+ and Illumination on Microtubule Dynamics in Light Responsive *Chlamydomonas*.

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The versatile microtubule system plays crucial roles in cells. Some cellular processes harness its signature dynamic instability, whereas others rely on stabilized microtubules. Its various properties are controlled by numerous proteins. A recent study using fluorescent plus end binding EB1-NeonGreen (NG) as a reporter shows that microtubules in flagellated green alga, *Chlamydomonas*, are highly sensitive to H^+, Na^+ and the illumination exciting NG. Light opens cation-permeant channelrhodopsins and modulates flagellar motility. To dissect individual effects, we investigated a β-tubulin mutant, *tub-2, which* was refractory to H^+-triggered rapid microtubule resorption, allowing revelations of the other changes. In *tub-2*, H^+ had imperfect biphasic effects. Both low and high H^+ decreased EB-NG plus end-binding comets, increased lattice binding and decelerated microtubule dynamics, albeit with distinct kinetics. In contrast, medium H^+ drastically increased comets numbers and piggyback incidences, at the expense of comet sizes and lattice binding. Low Na^+ had a similar effect as low H^+. However, when present together, their influences became muted. Notably, NG-exciting illumination accentuate ion-elicited changes. These results demonstrate how acidification, salination and the activity of channels and transporters could jointly shape this crucial cytoskeletal system and thus diverse organisms.

P1022/B146

Deciphering Tip Dynamics of *Drosophila Melanogaster* Class IV Neurons.

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Dendrites are fine processes that form branched arbors. These arbors serve as the neuron's antenna, receiving input and integrating signals from thousands of other neurons. Despite their central role in information processing by the brain, little is known about the molecular and cellular processes by which dendrites form and grow. My current research interest leads me to study the complex morphology of dendrites in Drosophila Class IV dendritic arborization cells, which is a good model cell for studying dendrite morphology. These cells grow in a highly dynamic manner. Their arbors reach diameters of about 500 µm after five days and contain up to 1000 branches. Yet, the individual dendritic tips, whose diameters are only 250 nm, grow and shrink at rates of a few microns per minute, which is roughly 100 times faster than the long-term growth of the neuron. Thus, the overall growth of the branched dendritic network in these cells results from a delicate balance between the formation of new tips, their growth, and their retraction. Currently, in the lab in collaboration with theoretician, we have developed a live cell imaging system, together with automated image analysis, to record and track the dendritic tips of Class IV cells. We observed that the dendritic tips stochastically switch between phases of growth (G), shrinkage (S) and pause (P) and can be fitted into a three-state model. The stochastic growth of dendritic tip is highly reminiscent of dynamic instability of microtubules.Microtubule, Microtubule-associated motors and other proteins (MAPs) play major roles in establishing and maintaining dendritic morphology. As a starting point for a systematic investigation of the role of motors and MAPs, I have used CRISPR to knock out the motor protein dynein specifically in Class IV cells using the established

technique. The key question is how this attenuated phenotype depends on the microscopic dynamics of the tips. By including the tip dynamic parameters measured in dynein mutants into the models, we hope to test whether this can account for the morphological phenotype, this approach will bridge the microscopic behavior of dendrite to the overall morphology of the cells which are necessary for linking genotype to phenotype.

P1023/B147

Alterations in Kif2c/mcak from Tumor Samples Increase Chromosome Instability.

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Recent years have seen the documentation of somatic mutations in human cancer in comprehensive databases such as the Catalogue of Somatic Mutations in Cancer (COSMIC). One limitation inherent in these databases is that, for many proteins, the effect of the recorded mutation on protein activity is unknown. The microtubule (MT) depolymerizing kinesis Kif2C/MCAK is often reported to be over expressed in certain cancers. The cellular effects of alterations in Kif2C/MCAK activity in cancer cells is an important question because increased Kif2C/MCAK activity has been reported to rescue chromosome instability (CIN), while decreased activity will increase CIN. Evaluation of the COSMIC database for alterations in Kif2C/MCAK activity reveal two principal, non-overlapping categories of Kif2C genetic changes: Point mutations and amplifications of the wild-type gene. We prepared Kif2C/MCAK constructs that identical to motor domain point mutation hotspots identified in the COSMIC database. We found that the majority of point mutations identified in the COSMIC database lead to impaired Kif2C/MCAK microtubule depolymerizing activity in cells. In contrast, we hypothesized that amplifications of Kif2C/MCAK would, presumably, increase cellular Kif2C/MCAK activity. We tested this hypothesis by quantifying the effect of point mutations and changes in Kif2C/MCAK levels on CIN by assaying lagging chromosomes during telophase. We prepared CRISPR modified transgenic cell lines that allowed us to rapidly remove endogenous GFP-FKBP-Kif2C/MCAK from the cytoplasm in Rapamycin. Relocalization of Kif2C/MCAK from microtubules and centromeres using this method led to an increase in CIN as quantified by lagging chromosomes. Cytoplasmic GFP-Kif2C/MCAK rescued this phenotype and restored proper chromosome segregation to cells in Rapamycin. In contrast, Kif2C/MCAK possessing mutations from hotspots identified in the COSMIC database exhibited high levels of CIN which became higher still, in combination with endogenous Kif2C/MCAK relocalization. Thus, while additional cytoplasmic GFP-Kif2C/MCAK could rescue CIN in cells with relocalized endogenous GFP-Kif2C/MCAK, we found that control cells expressing GFP alone were not tolerant of further increased Kif2C/MCAK activity. These data support the conclusion that both Kif2C/MCAK amplification and point mutations are likely to increase CIN in tumors in which they are identified.

P1024/B148

Clasp2 Mediates Interactions between Microtubules and Bundled Actin Filaments.

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Coordination between the microtubule and actin cytoskeletons is essential for cell motility, neuronal growth cone guidance, and wound healing. In a cellular context, this coordination is controlled by crosslinking proteins, and when disrupted can lead to disease. While studies in cells have identified important crosslinking proteins, the molecular mechanisms underlying the interactions between microtubules and actin filaments remain unclear. Members of the CLASP (Cytoplasmic Linker Associated

Protein) family of proteins have been implicated in cytoskeletal crosstalk. However, the specific dynamic interactions between microtubules and actin mediated by CLASPs remain less understood. Here, we use purified proteins to demonstrate direct interactions of CLASP2α with actin and microtubules, visualized using total internal reflection fluorescence microscopy (TIRFM). Our results demonstrate that CLASP2α directly interacts with actin in *vitro*, preferentially colocalizing with bundled F-actin, and is able to increase coalignment of microtubules along F-actin bundles. Furthermore, we characterized interactions between dynamic microtubules and bundled F-actin over a range of CLASP2α concentrations, and found that CLASP2α increases events of dynamic microtubule zippering along bundled F-actin. Our in *vitro* reconstitution approaches will elucidate the direct biochemical mechanisms underlying microtubule-actin coordination by CLASP2, essential for a range of cellular processes.

P1025/B149

Microtubule Minus-end Stability Is Dictated by the Tubulin Off-rate.

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Dynamic organization of microtubule minus ends is vital for the formation and maintenance of acentrosomal microtubule arrays. In vitro, both microtubule ends switch between phases of assembly and disassembly, a behavior called dynamic instability. Although minus ends grow slower, their lifetimes are similar to those of plus ends. The mechanisms underlying these distinct dynamics remain unknown. Here, we use an in vitro reconstitution approach to investigate minus-end dynamics. We find that minus-end lifetimes are not defined by the mean size of the protective GTP-tubulin cap. Rather, we conclude that the distinct tubulin off-rate is the primary determinant of the difference between plus- and minus-end dynamics. Further, our results show that the minus-end-directed kinesin-14 HSET/KIFC1 suppresses tubulin off-rate to specifically suppress minus-end catastrophe. HSET maintains its protective minus-end activity even when challenged by a known microtubule depolymerase, kinesin-13 MCAK. Our results provide novel insight into the mechanisms of minus-end dynamics, essential for our understanding of microtubule minus-end regulation in cells.

P1026/B150

Interplay between Microtubules and Integrin-mediated Adhesion in an giogenesis.

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Angiogenesis is the formation of new blood vessels from pre-existing vasculature and is essential during many physiological and pathological processes including wound healing and cancer. To initiate angiogenesis, cytokines from external sources activate endothelial cells (ECs) leading to increased extracellular matrix (ECM) degradation, cell migration, sprouting, and eventually reorganizing to form a tubular network of vessels. Changes in cell adhesion and shape, orchestrated by the cytoskeleton, are integral to the various steps of angiogenesis. Microtubules (MT) are major regulators of cell shape and are able to negatively regulate focal adhesion (FA) growth. This is evident after complete disruption of MT following nocodazole treatment which results in an increased number of myosin II-filaments and larger FA size. The interplay between MT and FA in the different stages of angiogenesis is not fully

understood. FA are associated with MT tips via proteins of KANK family, linking the FA protein talin with a complex of proteins trapping the MT ends. We have previously shown that depletion of KANK proteins led to an increase in FA size, similar to nocodazole treatment. Additionally, KANK proteins control the release/sequestration of GEF-H1, which sequentially affects Rho-Rho kinase (ROCK) signaling and actomyosin contractility. Here we demonstrate that in ECs (HUVEC), KANK1 localizes around FA complexes in close proximity to MT. RNAi knockdown of KANK1 induces the release of GEF-H1 from MT, resulting in elevated Rho-ROCK signaling and in turn an increase in myosin II-filaments and FA size, similar to treatment with nocodazole however without apparent disruption of MT. To study angiogenesis, we employed an *in-vitro* 3D microfluidic device that recapitulates the process of vascular sprouting. Additionally, we characterized the reorganization stage of angiogenesis by plating HUVECs onto an ECM support where the cells form capillary(tube)-like structures. In both systems we show that treatment of HUVEC with either nocodazole or siRNA depletion of KANK1 was sufficient to inhibit angiogenic sprouting or promote the disruption of tubular networks. We observe that co-knockdown of GEF-H1 or addition of ROCK inhibitor Y27632 was able to rescue the effects of KANK1 depletion, resulting in the restoration of vascular sprouts and capillary-like networks. Our research supports a unique role for KANK as a molecular tool to regulate MT/FA interactions, alter cellular contractility and inhibit angiogenesis, suggesting it may also possess a therapeutic potential.

P1027/B151

Dynein-mediated Nuclear Translocation of Yes-associated Protein through Microtubule Acetylation Controls Fibroblast Activation.

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Myofibroblasts are the major cell type that are responsible for increase the mechanical stiffness in fibrotic tissues. It has well documented that the TGF-β/Smad axis is required for myofibroblast differentiation under the rigid substrate condition. However, the mechanism driving myofibroblast differentiation in soft substrates remains unknown. In this research, we demonstrated that interaction of yes-associated protein (YAP) and acetylated microtubule via dynein, a microtubule motor protein drives nuclear localization of YAP in soft matrix, which in turn increased TGF-β1 induced transcriptional activity of Smad for myofibroblast differentiation. Pharmacological and genetical disruption of dynein impaired the nuclear translocation of YAP and decreased the TGF-β1 induced Smad activity even though phosphorylation and nuclear localization of Smad occurred normally in α-tubulin acetyltransferase (α-TAT1) knockout cell. Moreover, microtubule acetylation prominently appeared in the fibroblast-like cells nearby the blood vessel in the fibrotic liver induced by CCl_4 administration which were conversely decreased by TGF-β receptor inhibitor. As a result, quantitative inhibition of microtubule acetylation may be suggested as a new target for overcome the fibrotic diseases.

P1028/B152

Prc1 Is Required for Recruiting Eb1 to the Central Spindle Where Both Proteins Become Immobilized as the Midzone Compacts during Late Mitosis.

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At the onset of anaphase, antiparallel microtubules and associated proteins form a bundled structure known as the central spindle. How the dynamic properties of the central spindle develop as the cell transitions from metaphase to telophase is still poorly understood. To quantitatively characterize the

morphology and the dynamics of the central spindle in a native context, CRISPR/CAS9 endogenous gene editing was used in htert-RPE1 cells to fluorescently tag PRC1, a marker of antiparallel microtubule overlaps in the central spindle; and EB1, a marker for microtubule dynamics. FRAP analysis of mGFP-PRC1 in the central spindle shows that initially in metaphase the binding/unbinding turnover of PRC1 is fast, and then slows down in anaphase until PRC1 is essentially irreversibly bound to the midbody in telophase. In cells expressing both mGFP-EB1 and mCherry-PRC1 from their endogenous promotor EB1 tracks growing microtubule plus-ends, but surprisingly also colocalizes with PRC1 to the central spindle. Similar to PRC1, the turnover of mGFP-EB1 strongly decreases over time as the central spindle compacts and more PRC1 and EB1 accumulate there. These measurements demonstrate that the central spindle becomes 'solid' as it compacts. Central spindle localization of EB1 depends on the presence of PRC1, as shown by knock-down experiments. It also requires the ability of the C-terminal part of EB1 to interact with its binding partners, as shown by lentiviral expression of C-terminally mGFP-tagged EB1 that is known to disrupt the interaction with its partners. Taken together, these results reveal an unexpected link between PRC1 and EB1 during mitosis, although they have been thought until now to recruit their binding partners strictly to different localizations in human cells. Moreover, the turnover of both proteins indicate that the central spindle 'solidifies' as it ages, which may be an important part of its function.

P1029/B153

Microtubule Dynamics an alysis of Human Dermal Fibroblasts Using Hydrolyzed Eggshell Membrane-bound Acrylamide Gel Reflecting in Vivo Conditions.

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Cells change differentiation, proliferation, and metabolism in response to the environment. Hard plastic petri dishes that are routinely used in laboratories have unusual hardness that is incomparable to living organisms, and therefore change the properties of cells. In human fibroblasts, cells that have been passaged 30 times or more are used as senescent cell models, but detailed molecular mechanism is yet to be analyzed. We focus on cytoskeletal dynamics that control cell adhesion, especially microtubule dynamics. Based on previous studies, it has been shown that alphaB-crystallin, which functions as a tubulin / microtubule chaperone, controls cell shape and adhesion (PLoS One, 2016) and simultaneously found that microtubule dynamics are altered by knockdown and overexpression of alphaB-crystallin. On the other hand, cell culture systems that mimic the environment of living bodies have been actively constructed, and 2D and 3D collagen gels, laminin 511, decellularized ECM have been reported. We have developed and reported a cell culture system using chicken eggshell membrane, a unique natural ECM material. That is, the gene expression of human fibroblasts on hydrolyzed eggshell membranes conjugated to MPC polymer (Cell & Tissue Research, 2011) is surprisingly identical to the gene expression pattern when hydrolyzed eggshell membranes are applied to mouse skin (Cell & Tissue Research, 2019). This indicates that the hydrolyzed eggshell provides an ECM environment that reflects in vivo. However, since MPC polymers are expensive and difficult to obtain, we considered the use of versatile acrylamide gels. Transparent, microscopically observable thin gels with various amounts of hydrolyzed eggshell membranes were prepared using acrylamide, N, N'-methylenebis (acrylamide), and Acrylic acid N-hydroxysuccinimide ester. Human dermal fibroblasts were seeded on the gel and cultured for 24 hours. Thereafter, cells were fixed using formalin and the shape of the cells was observed by

immunostaining. In addition, RNA was extracted from the cells on the gel, and gene expression analysis was performed by RT-PCR. As a result, the expression patterns of type III collagen, Decorin, and MMP2 that are characteristic of eggshell membranes were confirmed, and it was found that they not only replace expensive MPC polymers but also agree with the results in mouse skin. This indicates that eggshell membrane conjugate acrylamide gel can be used as a cell culture system reflecting in vivo. In order to evaluate the health of fibroblasts, we investigated the relationship between spatiotemporal distribution of Vinculin controlled by alphaB-crystallin and microtubule dynamics using the developed eggshell membrane acrylamide gel system.

P1030/B154

Overexpression of α-tubulin Acetyltransferase 1 (αTAT1) Promotes Microtubule Hyperacetylation and Impairs Multiple Hepatic Polarized Protein Trafficking Steps.

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Alcoholic liver disease presents with a well-described clinical progression ranging from steatosis to fibrosis and ultimately cirrhosis. However, the mechanisms that promote alcohol-induced liver injury are far from established. The liver sustains the most damage from chronic alcohol consumption as it serves as the body's metabolic center. In this capacity, it converts alcohol into highly reactive metabolites that readily form adducts with proteins, lipids and DNA thereby disrupting liver homeostasis. It has also become apparent that chronic ethanol exposure induces post-translation protein modifications - most notably, lysine acetylation. Previously, we determined that microtubules are more acetylated in alcohol-treated hepatocytes, liver slices and in livers from ethanol-fed rats. We further correlated microtubule acetylation with many known defects in alcohol-induced protein trafficking including basolateral secretion, basolateral-to-apical transcytosis, STAT nuclear translocation and clathrin-mediated endocytosis. To determine whether microtubule acetylation itself can explain the alcohol-induced defects in protein trafficking, we overexpressed αTAT1 in polarized, hepatic WIF-B cells to promote tubulin acetylation in the absence of alcohol. Expression of αTAT1 led to a dose-dependent increase in tubulin acetylation up to 10-fold over control. In overexpressing cells with an equivalent increase in tubulin acetylation as seen in ethanol-treated cells (3 fold), we measured defects in secretion and transcytosis to similar levels as observed in ethanol-treated cells (50% of control). We further observed that the punctate basolateral distributions of markers of clathrin-mediated endocytosis (recycling receptors and AP2), were significantly increased in overexpressing cells suggesting that the dynamin-mediated fission of invaginated clathrin-coated pits was similarly impaired as we documented for ethanol-treated cells. Somewhat surprisingly, STAT5 nuclear translocation was not impaired in αTAT1 expressing cells which may point to differences in the mechanism regulating this process. We conclude that microtubule hyperacetylation is the underlying feature of a subset of alcohol-induced defects in protein sorting and further suggest that agents already in clinical trials that modulate cellular acetylation may provide a novel therapeutic for chronic alcoholics.

P1031/B155

Measurement and Modeling of Microtubule Tip Dynamics.

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The ability of microtubules to undergo dynamic instability is a fundamental process in all cells and a target of antimitotic drugs. Traditionally, microtubule growth and shrinkage phases in vitro have been assessed through kymograph analysis, which provides limited insight into the precise dynamics at growing tips. Here, we used interference reflection microscopy (IRM) to image dynamic microtubules at 10 frames/s over 10s of minutes, and used a half-gaussian function to track the precise position of the growing plus- and minus-end positions. To study polymerization dynamics occurring during phases of growth and shrinkage, time-dependent fluctuations of tip position were fit to an equation for drift plus diffusion. We found that the plus-tip diffusivity and velocity both increase with increasing tubulin concentration. To understand tubulin on-and off-kinetics, we measured tip diffusivity, growth rate, shrinkage rate, and microtubule lifetimes, and incorporated them into a biochemical model. Using our measured parameters for both the plus- and minus-ends, we were able to put constraints on the on-rate, the longitudinal bond energy, and the lateral bond energy parameters. Current work is focused on comparing the growth of microtubules in GTP versus the non-hydrolyzable analog GMPCPP. Collectively, our measurements will be utilized to understand the impact of hydrolysis on reversible tubulin binding at the plus- and minus-ends.

P1032/B156

Electron Cryo-tomography and Brownian Dynamics Modeling Elucidate the Mechanism of Microtubule Polymerization.

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Recent studies from our labs have shown that the ends of both growing and shortening microtubules (MTs) terminate in bent protofilaments (PFs), suggesting that MTs polymerize by the addition of bent GTP-tubulin to the tips of curving PFs (McIntosh et al., J. Cell Biol., *217*:2691(2018)). Here we develop a Brownian dynamics model for MT growth by examining a range of values for all key parameters that are not specified by observation. We then use the model to make predictions about MT assembly under a range of conditions. The model predicts that the shapes of growing MT tips should be insensitive to growth rate. We have tested this prediction by varying the soluble tubulin concentration over 4X, by adding two concentrations of a TOG polymerase that increases MT growth rate, and by slowing MT growth with low concentrations of Taxol or Epothilone. All results conform to model predictions. The model predicts that the structures of adjacent PFs should not correlate, compared with randomly chosen PFs from a MT tip, and this prediction too is valid. The model predicts that the irregularity of multiple PFs at each MT tip, or raggedness, should not vary with the rates of MT growth or shortening. This prediction is approximately true, but experimental results show greater tip raggedness than model MTs with only two exceptions: shortening MTs and those growing in GMPCPP. We surmise that the rates of individual PF elongation vary for reasons not included in the current model. One possible explanation is the heterogeneity of brain tubulin, so we examined the tips of MTs elongating with a human tubulin, purified from an embryonic kidney cell line, which is composed predominantly of one

isoform of α-tubulin and two of β, and which harbors no detectable posttranslational modifications. This tubulin also produced ragged MT ends. Ragged ends in model MTs can be obtained when the tubulin addition rate constant is lower than we have commonly used, making it as low as that recently measured for yeast tubulin. This may explain why our current model is less successful in accounting for raggedness than other features of MT growth.

P1033/B157

A Kink in Brain Development: Altering Tubulin's Conformational Transitions Disrupts Neuronal Migration.

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Neurons require distinct microtubule activity in order to migrate properly, and therefore it is crucial that microtubule dynamics are tightly regulated throughout the neuron. Lissencephaly is a neurodevelopment disease that results from the improper migration of neurons in the developing brain and has been linked to a variety of mutations in the *TUBA1A* gene. Despite the association between *TUBA1A* mutations and disrupted neuronal migration, we still do not understand how tubulin modifications impact greater neuronal cell biology. In its free state, the tubulin heterodimer is in a kinked conformation, but must subsequently straighten as it is assembled into microtubule polymer. This transition from a kinked-to-straight conformation is therefore a potential point of microtubule dynamics regulation. Located at the position where the tubulin heterodimer hinges between its kinked and straight conformations is an understudied region of α-tubulin, the H11' helix. Our preliminary computational modeling indicates that mutating a single residue in the α-tubulin H11' helix perturbs the kinked conformation of the tubulin heterodimer, indicating the importance of this helix in establishing a kinked state. Interestingly, two patients with lissencephaly have been identified to have missense mutations within the α-tubulin H11' helix. Both patients have mutations that alter the valine at residue 409 (V409) of *TUBA1A*, but the mutations and resulting phenotypes are distinct. One patient with a V409A substitution exhibits severe lissencephaly, whereas another with a V409I mutation exhibits a milder form of pachygyria. We find that the ectopic expression of either *TUBA1A*-V409A or -V409I dominantly disrupts neuronal migration in the developing mouse brain, reminiscent of the phenotype we observe in patients. To determine the underlying molecular mechanisms of the *TUBA1A*-V409A and -V409I mutants that disrupt neuronal migration, we modeled these mutants in budding yeast. We show that both mutants have increased rates of microtubule polymerization and depolymerization. However, microtubules composed of either of these two mutants have depleted localization of XMAP215/Stu2, which selectively binds kinked heterodimer, to the microtubule plus-end. Decreased localization may therefore indicate a loss of kinked tubulin that XMAP215/Stu2 can readily bind. Taken together, these data indicate that V409 mutants in α-tubulin disrupt the regulation of microtubule dynamics that is normally coordinated via XMAP215/Stu2. We propose that these effects are attributable to a disruption of the kinked tubulin conformation. Our work thus highlights a critical role for the proper regulation of tubulin conformation in migrating neurons.

P1034/B158

The Role of Kif19 in Cell Migration, Microtubule, and Focal Adhesion Dynamics.

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Focal adhesions are a critical component of cell migration and attachment. Dysregulation and decreased stability of focal adhesions have been linked with cancer invasiveness and metastasis. It is unclear how focal adhesion disassembly occurs, but there is a growing body of work indicating that microtubules, and specifically their high dynamicity at adhesions, are a key regulator. While studies have described several mechanisms for guiding microtubule growth towards focal adhesions and mediating adhesion disassembly, the regulation of microtubule depolymerization at focal adhesions remains unknown. We have identified a member of the kinesin-8 superfamily, Kif19, as a protein that regulates cell migration and may destabilize microtubules at focal adhesions. Kif19 has previously been shown to move along and depolymerize microtubules in vitro. Our preliminary studies have demonstrated that depletion of Kif19 results in a significant inhibition of cell motility and decreased microtubule catastrophe at focal adhesions. Thus, we propose that Kif19 is a positive regulator of cell migration that acts by localizing to focal adhesions and depolymerizing nearby microtubules. Here we show that Kif19 depletion results in a significant increase in focal adhesion area, as well as a significant change in cell shape to a more circular morphology with fewer actin protrusions. Additionally, Kif19 depletion results in an increased proportion of tyrosinated (i.e. dynamic) microtubules compared to total tubulin. Our data imply that Kif19 is important in regulating focal adhesion disassembly and microtubule dynamics and may be important for regulating cell morphology.

P1035/B159

Investigating the Effect of Novel Microtubule-Severing Enzyme Fidgetin-Like 2 on Rac1 and RhoA GTPase Activation at the Leading Edge.

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Fidgetin-like 2 (FL2) is a recently characterized microtubule-severing enzyme, which reduces cell motility by severing dynamic microtubules at the leading edge of migrating cells. We hypothesize that FL2 KD leads to a shift in local RhoA and Rac1 activation kinetics, which may then influence downstream effectors in order to suppress cell migration. Both Rac1 and RhoA GTPases are involved in key cell motility pathways, and both can be regulated by microtubule dynamics. Therefore, we used FRET (Forster resonance energy transfer) imaging with either a Rac1 or RhoA biosensor to investigate changes in activation after FL2 KD. Our data show that RhoA activation kinetics are spatially polarized after FL2 depletion, while typical Rac1 activation kinetics are simply strengthened. These experiments begin to provide some insight into the mechanism by which FL2's local microtubule-severing activity may suppress cell motility.

Microtubule Nucleation and Organization

P1036/B160

Microtubule Nucleation in Mouse Bone Marrow-derived Mast Cells Regulated by Src Homology 2 Domain-containing Protein Tyrosine Phosphatase 1.

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Microtubules (MT) play an important role in essential cellular events. Rapid and transient reorganization of MTs was observed upon antigen-induced activation of bone marrow-derived mast cells (BMMCs), key components of adaptive and innate immune responses. Propagation of signals from activated cell membranes results in the generation of protrusions containing MTs, degranulation, release of multiple inflammatory mediators. While MTs are known to be important for degranulation, the molecular mechanisms responsible for microtubule reorganization remain largely unknown. In BMMCs, MTs are nucleated from the centrosomes, where γ-tubulin complexes are essential for this process. We have recently found that Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 1 (SHP-1; Ptpn6) form complexes with γ-tubulin complex proteins. Therefore, we aim to test the hypothesis that SHP-1 can influence MT nucleation by following MT regrowth in BMMCs. We have developed a user-friendly macro for rapid processing of MT regrowth raw images using Fiji software (Klebanovych et al, Cells 8, e345, 2019). When compared to wild type cells, MT regrowth experiments revealed large MT asters emanating from interphase centrosomes and centrosomal accumulation of γ-tubulin in cells lacking SHP-1. Moreover, the inhibition of the SHP-1 activity by inhibitors TPI-1 and NSC87877 augmented MT nucleation as well. Further experiments with antigen-activated cells showed that the deletion of SHP-1 stimulated both the generation of microtubule protrusions and the degranulation. Our data suggest that SHP-1 is a novel negative regulator of MT nucleation that affects γ-tubulin level in centrosomes of BMMCs.

P1037/B161

Protein Tyrosine Phosphatase SHP-1 Is a Regulator of Microtubule Organization in Activated Mast Cells.

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Antigen-mediated activation of mast cells initiates signaling events leading to degranulation and release of inflammatory mediators. While rearrangement of cytoskeleton is essential for mast cells degranulation, molecular mechanisms that modulate microtubule reorganization during this process are not fully understood. Microtubule nucleation is mediated by γ-tubulin complexes. Rapid tyrosine phosphorylation of proteins interacting with γ-tubulin in activated mast cells has been described (Sulimenko et al., 2006). Here, we report on interaction of Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 1 (SHP-1; *Ptpn6*) with γ-tubulin complex proteins and its regulatory role in microtubule organization in bone marrow-derived mast cells (BMMCs). Reciprocal immunoprecipitation experiments revealed complexes of SHP-1 with γ-tubulin, GCP proteins and protein tyrosine kinase Syk. Pull-down experiments with truncated forms of γ-tubulin and SHP-1 unveiled corresponding interaction domains. When compared to controls, activated SHP-1 depleted cells had higher level of tyrosine phosphorylation, enhanced degranulation, as well as increased expression of cytokines and

prostaglandins. Depletion of SHP-1 also resulted in stimulation of de novo microtubule nucleation and generation of protrusions containing microtubules. Our data suggest a novel SHP-1 dependent mechanism for the suppression of microtubule formation in later stages of mast cell activation.

P1038/B162

Molecular Mechanism and Kinetics of Microtubule Nucleation in the Cell.

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A variety of cytoskeletal structures are formed by regulating where and when microtubules are nucleated. How microtubules are generated from αβ-tubulin dimers in the cell? This has been a major open question in understanding how the cytoskeleton is assembled ever since the discovery of tubulin in the 1960s. Even though the γ-tubulin ring complex (γ-TuRC) has been accepted as the universal microtubule nucleator of the cell for several decades, its mechanism as well as its regulation in cytosol still remains to be elucidated. Partly, this has been due to the inability to purify the 2.2 MDa γ-TuRC consisting of roughly 40 subunits in biochemical quantities, such that to date no live assay for MT nucleation by γ-TuRC has been reported. Here, we overcome this hurdle by using purifying γ-TuRC from Xenopus egg extract, with which we reconstitute γ-TuRC mediated MT nucleation in vitro. Most importantly, we establish a novel assay in which single γ-TuRC molecules are observed live at the single molecule level to study MT nucleation. We find that γ-TuRCs display a kinetic lag in microtubule nucleation and a critical size of 4-5 tubulin dimers are needed for successful nucleation event, thereby defining the actual 'nucleus'. We demonstrate that the lag in γ-TuRC nucleation is the result of weaker interactions between γ-tubulin and αβ-tubulin, compared to strong interations between αβ-tubulin dimers. This is the molecular basis of the nucleation barrier. The recently discovered co-nucleation factor XMAP215 accelerates γ-TuRC nucleation by decreasing the critical concentration of tubulin at which γ-TuRCs generate microtubules. Single molecule experiments with 3 fluorescently components -γ-TuRC, XMAP215 and tubulin- show that XMAP215 and γ-TuRC associate first followed by generation of a microtubule from the nucleation site. Strikingly, microtubule associated proteins that tune the microtubule catastrophe and rescue frequency, TPX2 and EB1, unexpectedly had no effect on microtubule nucleation by γ-TuRC. In summary, in this work we define the nucleation barrier for MT nucleation by γ-TuRC and reveal its molecular basis. Nucleation by γ-TuRC is driven by finely tuned interactions at the interface of γ-TuRC to the microtubule and turned by microtubule associated proteins.

P1039/B163

Identifying and Understanding the Role of Microtubule Associated Proteins in Differentiated Tissue.

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Microtubules are intracellular polymers that are required for various essential processes ranging from cell division to maintaining cell structure. Underlying the ability of microtubules to contribute to these diverse functions is the cell's ability to control microtubule organization, nucleation, and dynamics, which in part is achieved by microtubule associating proteins (MAPs). MAPs can affect microtubules in numerous ways including by stabilizing or destabilizing individual polymers or by creating higher order arrays by crosslinking them together. While some MAPs have been characterized, the diversity of MAPs between cell types in *vivo* and how these differences affect microtubule dynamics and organization is

not well understood. We are using *C. elegans* as a model to identify novel MAPs required for essential functions in differentiated cells. Using a published protocol for MAP isolation (Aamodt et al. 1989), we have purified proteins from mixed-stage worm lysates that co-sediment with taxol-stabilized microtubules. Using mass spectrometry, we have identified 1378 potential MAPs, of which 655 have been annotated by other groups' depletion studies as being required for embryogenesis. We are using a bioinformatic approach to further refine this list in order to guide a candidate based RNAi screen for genes that are important for microtubule organization and dynamics. Our biochemical approach revealed that MAPH-1.1, 1.2, and 1.3, the *C. elegans* orthologs of MAP1 (Waaijers et al. 2016) are among the mostly highly enriched MAPs in our dataset. The MAP1 family has been primarily studied in neurons, but little is known about how it interacts with microtubules or its function in other tissues. Unlike other MAP1 family members, C. elegans MAPH-1.1 is known to be expressed in many more tissues including the hypodermis and the muscles (Waaijers et al. 2016). Depletion of MAPH-1 suggest an important role of the MAP1 family in development, but its role in patterning and organizing microtubules is unknown. We show a novel localization of MAPH-1.3 to the microtubules of the mitotic spindle. We also show that MAPH-1.1 directly binds the entire length of the microtubules in vitro and may play a role in promoting growth. Our data suggests that MAPH-1 may play a role in microtubule patterning and may provide insight into the role of MAP1 proteins in non-neuronal tissue.

P1040/B164

Conserved Mechanisms Driving Branched Cytoskeletal Network Growth in the Rhizarian Syncytial Amoebae *Filoreta Ramosa*.

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Multicellularity involving cell organization and differentiation generates emergent morphological complexity through the development of specialized cytoskeletal structures and cell types. Multicellularity has evolved at least five times across the eukaryotic supergroups, including members of the SAR (Stramenopiles/Alveolates/Rhizaria). Our recently isolated Rhizarian amoeba, *Filoreta ramosa*, exhibits "aggregative multicellularity". Individual amoebae fuse and form a complex multinucleate syncytium, organized and structured by the cytoskeletal network of branching and anastomosing pseudopodia. Through branching mechanisms reminiscent of neuronal growth cones and dendritic arborization, the network can grow to span several centimeters. Self-recognition enables the fusion of neighboring syncytial branches, forming a dynamic and self-repairing complex morphology. How and when have the cytoskeletal mechanisms enabling polarized growth cones and branching evolved in Eukarya? We are using our recently completed genome combined with super-resolution imaging and morphometric analyses to quantify cytoskeletal elements of complex network growth and development. We used antibodies raised specifically to *F. ramosa* ɣ-TURC components and EB1 to visualize non-centrosomal MT nucleation and polarity throughout the network. We also compared morphological perturbations in the network following treatment with cytoskeletal drugs affecting actin and MT dynamics. As in neuronal branches, the branched network is organized with longitudinal MT arrays and actin-rich pseudopodial protrusions initiate branch formation. Cytoskeletal drugs alter syncytial network development, underscoring the critical role of MT and actin dynamics and interactions in generating the complex branched morphology in Rhizaria and Metazoa. We predict that the mechanisms governing the intricate cytoskeletal networks in *F. ramosa* are emergent properties of simple branch and anastomosis patterns involving conserved cytoskeletal proteins. Thus, the extensions and branching mechanisms

typified by neuronal growth cones are conserved in Rhizaria and pre-date the divergence of major eukaryotic lineages.

P1041/B165

Direct Observation of Branching MT Nucleation in Living an imal Cells.

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Centrosome-mediated microtubule (MT) nucleation has been well-characterized; however, numerous non-centrosomal MT nucleation mechanisms exist. The branching MT nucleation pathway envisages that the γ-tubulin ring complex (γ-TuRC) is recruited to MTs by the augmin complex to initiate nucleation of new MTs. While the pathway is well-conserved at a molecular and functional level, branching MT nucleation by core constituents has never been directly observed in animal cells. Here, multi-color TIRF microscopy was applied to visualize and quantitatively define the entire process of branching MT nucleation in dividing *Drosophila* cells during anaphase. The steps of a stereotypical branching nucleation event entailed augmin binding to a "mother" MT, recruitment of γ-TuRC after 15s, followed by nucleation 16s later of a "daughter" MT at a 36° branch angle. Daughters typically remained attached throughout their ~40s lifetime unless the mother depolymerized past the branch point. Assembly of branched MT arrays, which did not require D-TPX2 (*Drosophila* TPX2), enhanced localized RhoA activation during cytokinesis.

P1042/B166

Pcm Proteins Have Various Requirement during Development, between Cell Division and Ciliogenesis in C. Elegans.

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The centrosome acts as a microtubule organizing center (MTOC) during mitosis, forming radial arrays essential to separate cellular components between daughter cells. Microtubules are organized at the centrosome by pericentriolar material (PCM) complexes. The fate of the centrosome following mitosis varies by cell type and developmental context. In differentiated cells, the centrosome is inactivated as an MTOC, losing its PCM and associated microtubules, and MTOC function is reassigned to other cellular sites. In contrast, in some types of ciliated cells the centrioles at the heart of the centrosome are repurposed as basal bodies to template the assembly of cilia and maintain some microtubule nucleation activity (Kunimoto et al. 2011, Clare et al. 2017, Roque et al. 2018). The exit from the cell cycle is thus not enough to explain the inactivation of the centrosome upon differentiation, and mechanisms controlling centrosomal MTOC function remains largely uncharacterized in differentiated cells. We are using *C. elegans* as a model to characterize MTOC recruitment and regulation at the centrosome in differentiated cells. Surprisingly, we have found that PCM proteins are differentially localized and have differential requirement in MTOC function depending on cell type and location. First, we found that PCM proteins in mitotic cells separate into overlapping spheres surrounding the centrioles with known binding partners such as the two main scaffolding protein SPD-2/CEP192 and SPD-5 separating into an inner and an outer sphere, respectively (Magescas et al 2019). Second, we found that although both SPD-2 and SPD-5 are essential for the first mitotic division in *C. elegans*, using tissue specific degradation we found that SPD-2 is dispensible for later divisions such as of the embryonic intestinal cells. Third, we found that SPD-5 and the microtubule nucleating complex γ-TuRC but not SPD-2 localize to the base of cilia. This result is particularly surprising given that basal bodies in *C. elegans*are degraded upon

ciliogenesis and suggests that these centriole-less remnants of the PCM might be required for ciliogenesis and/or MTOC function. Indeed, SPD-5 depletion in ciliated neurons resulted in aberrant ciliogenesis. Based on our data we propose that the PCM is composed of distinct SPD-5 based subcomplexes that can be differently regulated to impart MTOC function.

P1043/B167

Aurora a Site Specific TACC3 Phosphorylation Regulates Astral Microtubule Assembly by Stabilizing γ-Tubulin Ring Complex.

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Astral microtubules emanating from the mitotic centrosomes play pivotal roles in defining cell division axis and tissue morphogenesis. Previous studies have demonstrated that human transforming acidic coiled-coil 3 (TACC3), the most conserved TACC family protein, regulates formation of astral microtubules at centrosomes in vertebrate cells by affecting γ-tubulin ring complex (γ-TuRC) assembly. However, the molecular mechanisms underlying such function were not completely understood. Here, we show that Aurora a site-specific phosphorylation in TACC3 regulates formation of astral microtubules by stabilizing γ-TuRC assembly in human cells. Mutation of the most conserved Aurora a targeting site, Ser 558 to alanine (S558A) in TACC3 results in robust loss of astral microtubules and disrupts localization of the γ-tubulin ring complex (γ-TuRC) proteins at the spindle poles. Under similar condition, phospho-mimicking S558D mutation retains astral microtubules and the γ-TuRC proteins in a manner similar to control cells expressed with wild type TACC3. Time-lapse imaging reveals that S558A mutation leads to defects in positioning of the spindle-poles and thereby causes delay in metaphase to anaphase transition. Biochemical results determine that the Ser 558- phosphorylated TACC3 interacts with the γ-TuRC proteins and further, S558A mutation impairs the interaction. We further reveal that the mutation affects the assembly of γ-TuRC from the small complex components. The results demonstrate that TACC3 phosphorylation stabilizes γ- tubulin ring complex assembly and thereby regulates formation of centrosomal asters. They also implicate a potential role of TACC3 phosphorylation in the functional integrity of centrosomes/spindle poles.

P1044/B168

Theory of Crosslinker-mediated Mitotic Spindle Assembly.

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Cells grow, move, and respond to outside stimuli by large-scale cytoskeletal reorganization. A prototypical example of cytoskeletal remodeling is mitotic spindle assembly, during which microtubules nucleate, undergo dynamic instability, bundle, and organize into a bipolar spindle. Key mechanisms of this process include regulated filament polymerization, crosslinking, and motor-protein activity. Remarkably, using passive crosslinkers, fission yeast can assemble a bipolar spindle in the absence of motor proteins. We develop a torque-balance model that describes this reorganization due to dynamic microtubule bundles, spindle-pole bodies, the nuclear envelope, and passive crosslinkers to predict spindle-assembly dynamics. We compare these results to those obtained with kinetic Monte Carlo-Brownian dynamics simulations, which include crosslinker-binding kinetics and other stochastic effects.

Our results show that rapid crosslinker reorganization to microtubule overlaps facilitates crosslinker-driven spindle assembly, a testable prediction for future experiments. Combining these two modeling techniques, we illustrate a general method for studying cytoskeletal network reorganization.

P1045/B169

Investigation of Nucleation Independent Functions of the γ-TuRC.

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Microtubules play essential roles in various cellular activities, including intracellular transport, cell motility, and cell division. For microtubules to carry out these functions, their formation and organization must be tightly regulated. This regulation is imparted by the multimolecular γ-tubulin ring complex (γ-TuRC), which according to current models nucleates microtubules and remains bound to microtubule minus ends after polymer formation as a “cap” and anchor point, contributing to the stabilization of functional microtubule arrays. However, it has been difficult to dissect how the γ-TuRC functions in each of these different activities, especially in cell-based contexts. To differentiate between γ-TuRC nucleating and non-nucleating functions, we developed a γ-tubulin knockdown (KD) and a KD/addback human cell line which expresses mutant γ-tubulin that is GTP-binding deficient and has been shown in yeast to be compromised in its nucleation activity. We found that the interphase microtubule array was minimally affected by γ-tubulin KD or mutant expression. In contrast, >75% of mitotic cells in both the KD and KD/mutant-addback conditions formed monopolar spindles, and the mitotic index was increased ~4-fold. Surprisingly, microtubule number did not seem to decrease in these cell lines. Further investigation of these cell lines using advanced microscopy techniques coupled with in vitro systems will allow us to determine a separation of function between γ-TuRC nucleating and nucleation independent activities.

P1046/B170

Testis-specific Gamma-tubulin Complex Protein Paralogs with a Specialized Function in *Drosophila* Spermiogenesis.

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The γ-Tubulin Ring Complex, or γ-TuRC, is a highly conserved microtubule (MT) nucleation complex comprised of γ-tubulin and γ-Tubulin Complex Proteins (GCPs). GCP2-GCP3 heterodimer interactions are facilitated by the grip1 motif and each GCP binds to γ-tubulin through a grip2 motif. This tetrameric γ-tubulin small complex (γ-TuSC) oligomerizes to form the γ-TuRC, consisting of seven γ-TuSC subcomplexes. The γ-TuRC is organized into a lockwasher-shaped ring, such that 13 γ-tubulin molecules are positioned apically to anchor/nucleate microtubule (MT) assembly by binding to the α-tubulin subunit of αβ-tubulin dimers and initiating the assembly of 13 protofilaments into a MT. γ-TuRCs temporally and spatially mediate MT nucleation via interactions with regulators and site-specific anchors. For example, at centrosomes, the major MT organizing centers in animal cells, γ-TuRCs are the chief microtubule nucleators and are required for proper cell division. As a result, GCP2 and GCP3 are essential in *Drosophila melanogaster*. In general, γ-TuRCs are highly conserved throughout eukaryotes and no heterogeneity in the GCP core complex has been described. Here we describe paralogs of GCP2 and GCP3 that appear to be expressed exclusively in the testes of *Drosophila*. We have named these proteins GCP2t and GCP3t (“t” for testis). Our objective is to determine if GCPt paralogs have a diverged function from the canonical γ-TuRC. Our results suggest that GCPt paralogs have a specialized function

that is required for spermiogenesis. The 'conventional' GCP2 and GCP3 proteins are also expressed in testis, and mutations in those genes disrupt cell division. In contrast, mutations in the *GCP2t* and *GCP3t* do not impact cell division, but impair spermiogenesis, resulting in spermatozoa that are immotile and fail to enter the seminal vesicle. Remarkably, GCP2t is absent from centrosomes in spermatocytes where GCP2, GCP3, and γ-tubulin play a key role in meiosis. Moreover, ectopically-expressed GCP2t failed to localize at centrosomes, suggesting that it does not form a complex with conventional GCP3. In addition, GCP2t fails to localize to the mitochondrial MTOC of post-meiotic spermatids, previously described by our lab, that activates γ-TuRC nucleation via the CM1 domain of CnnT, a testis-specific splice variant of centrosomin. Rather, GCP2t co-localizes with γ-tubulin at a unique and poorly understood MTOC called the centriolar adjunct in post-meiotic spermatids. Mutants in *gcp2t* and *gcp3t* block γ-tubulin localization to the centriolar adjunct. From our current data, we hypothesize that these GCP paralogs form a testis-specific γ-TuRC at the centriolar adjunct with a noncanonical function that is essential for sperm maturation.

P1047/B171

The Mitotic Motor Kifc1 Is Crucial for the Organization of the Microtubule Cage Around the Nucleus during Neuronal Migration.

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During neuronal migration, most but not all microtubules are attached to the centrosome. Dynein-based forces tug on the centrosome-attached microtubules that extend into the leading process to drag the centrosome along with the nucleus as the neuron itself moves. Crucial to this process is the organization of a portion of the microtubules into a cage around the nucleus, which enables the nucleus to rotate as it is dragged along. Here we investigated a potential role in neuronal migration of KIFC1, a molecular motor that we recently showed has important functions in the axon to organize microtubules and thereby regulate axonal growth and retraction. KIFC1 belongs to the kinesin-14 sub-family of kinesins, which are the only kinesins that move toward minus ends of microtubules rather than plus ends. Unlike most miotic kinesins, KIFC1 is strongly expressed throughout the life of the neuron. Here we report studies on cultured rat cerebellar migratory neurons either depleted of KIFC1 with siRNA or treated with AZ82, a drug that inhibits KIFC1. The results indicate that when KIFC1 is depleted or inhibited, the migration of the soma is impaired, with the nucleus failing to rotate and translocate forward as it normally does. Pulldown experiments indicate that KIFC1 interacts with proteins in the nuclear membrane. The behavior of GFP-tagged EB3 (which fluorescently marks the plus ends of microtubules) in the soma indicates that when KIFC1 is inhibited or depleted, microtubules no longer form a proper cage around the nucleus. In *vivo* studies in mice show that KIFC1 depletion by shRNA results in neurons with morphological defects, as well as delayed migration to the cortical plate. We, therefore, propose that KIFC1 is involved in nucleokinesis in migratory neurons by tethering microtubules to the nucleus in a manner crucial for the structure of a microtubule cage that allows for nuclear rotation. Together with our previous observations on KIFC1's role in the axon, we conclude that KIFC1 takes on distinct duties at different stages of the life of the neuron.

P1048/B172

A Role for the Apical PAR Complex in Reorganizing Microtubules in Dividing Intestinal Cells.

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Dividing and differentiating cells require different arrangements of microtubules to function. Mitotic cells establish centrosomes as microtubule organizing centers (MTOCs), producing radial microtubule arrays that are critical for chromosome segregation. In contrast, polarized epithelial cells form parallel arrays of microtubules emanating from a non-centrosomal MTOC, the apical membrane, that promote cell polarity and intracellular transport. During development and tissue homeostasis, some polarized epithelial cells divide, presenting an important but poorly understood obstacle: microtubules must temporarily cycle between the apical surface and the centrosomes. The developing *C. elegans* intestine provides an excellent in *vivo* epithelial model to study how this microtubule reorganization is achieved. After the 16-cell embryonic intestine polarizes and establishes an apical MTOC, exactly four "E16*" cells divide again. The E16* divisions involve a rapid change in microtubule organization from apical to centrosomal as cells enter mitosis, and back to apical upon mitotic exit. Using fluorescent markers, genetic screens, and tissue-specific protein depletion with live imaging, we are testing the hypothesis that apical polarity proteins control microtubule reorganization during the E16* divisions. During mitosis, we observe that, like microtubules, MTOC-associated proteins also leave the apical membrane as the centrosome becomes the MTOC. However, the apical PAR polarity proteins remain at the apical membrane during the E16* divisions, suggesting that they may act as a memory mark and help direct the return of microtubules and MTOC proteins after division. Consistent with this model, we have found that intestine-specific depletion of the apical polarity proteins PAR-6 and PKC-3 disrupts apical MTOC reformation following the E16* division. A pilot forward genetic suppressor screen has isolated a suppressor of the MTOC defects caused by PAR-6 depletion. These experiments reveal a role for PAR proteins in returning MTOC function to the apical membrane following mitosis, a critical step in epithelial cell divisions across organisms.

P1049/B173

Biochemical Reconstitution of Branching Microtubule Nucleation.

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The microtubule cytoskeleton supports cell function by giving cells their shape, organizing their interior and segregating chromosomes. Microtubules are nucleated from specific locations at precise times in the cell cycle, and several of these microtubule nucleation pathways converge to form a particular architecture in the cell. Although many microtubule nucleation pathways have been identified, it remains poorly understood which factors comprise them and how they shape the microtubule cytoskeleton. Here, using purified proteins we biochemically reconstitute branching microtubule nucleation, a nucleation pathway where microtubules originate from pre-existing microtubules, which is essential for spindle assembly and chromosome segregation in many cell types and species. We found that besides the microtubule nucleator gamma-tubulin ring complex (γ-TuRC), the two branching effectors augmin and TPX2 are required to efficiently nucleate new microtubules from pre-existing ones. TPX2 has a key role in this process, as its binding sites along the microtubule generate regularly-spaced patches that serve as the sites of microtubule nucleation by recruiting augmin and γ-TuRC. We found that the preferred branching angle was less than 90 degrees, and the absence of TPX2 resulted in shallower branch angles. Our results demonstrate how the microtubule nucleator γ-TuRC is brought to

its site of nucleation to cause branching microtubule nucleation, a process critical for spindle assembly and axon architecture. We anticipate our results to help explain how other microtubule nucleation pathways give rise to a cell's microtubule cytoskeleton. Furthermore, our work serves as a stepping stone to reconstitute complete microtubule structures such as the mitotic spindle and to engineer specific microtubule architectures in health and disease.

P1050/B174

***In Vivo* Proximity Labeling of PTRN-1/Patronin with TurboID Reveals Novel Components of Non-centrosomal MTOCs in Epithelial Cells.**

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Microtubules have dynamically growing plus ends and comparatively stable minus ends that are localized to microtubule organizing centers (MTOCs). The centrosome is the primary MTOC during cell division, but in differentiated cells, MTOC function is often reassigned to non-centrosomal sites (ncMTOCs). Unlike the centrosome, the components that are necessary to grow and localize microtubules at ncMTOCs are poorly understood. To address this gap in knowledge, we are identifying additional ncMTOC components using proximity labeling, a technique that can identify proximal protein networks in *vivo*. In particular, we developed TurboID, a promiscuous BirA mutant enzyme that rapidly catalyzes the covalent attachment of biotin to proximal proteins. Proteins tagged with biotin are then isolated and identified by mass spectrometry. To identify novel ncMTOC components, we fused TurboID to the microtubule minus end protein PTRN-1/Patronin and expressed this transgene in *C. elegans* embryonic intestinal epithelial cells. This strategy resulted in spatially restricted biotinylation at the apically localized ncMTOC and a list of 62 candidate ncMTOC components including the spectraplakin VAB-10B, which we verified as a PTRN-1 physical interactor by immunoprecipitation. Using tissue-specific degradation and mutant analysis of VAB-10B, we found that VAB-10B orchestrates microtubule localization during ncMTOC establishment, which is helping us understand the mechanisms by which microtubules are initially targeted to an ncMTOC in vivo. In addition, we find a role for VAB-10B in targeting PTRN-1 to the ncMTOC and are exploring the role of VAB-10B in targeting other microtubule-associated proteins. As the apical polarity protein PAR-3 is properly localized following VAB-10B depletion, we propose a model where VAB-10B serves as a scaffold to couple microtubule organization to polarity determinants. In addition to VAB-10B, we are currently testing the ncMTOC-related functions of other candidates from our proximity labeling dataset and will present our results.

P1051/B175

Growth Cone-localized Microtubule Organizing Center Determines Microtubule Orientation in Dendrites.

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A polarized arrangement of neuronal microtubule arrays is the foundation of membrane trafficking and subcellular compartmentalization. Axons contain exclusively "plus-end-out" microtubules while dendrites contain a high percentage of "minus-end-out" microtubules. Here we show that the dendritic growth cone contains a non-centrosomal microtubule organizing center (ncMTOC), which generates minus-end-out microtubules along outgrowing dendrites and plus-end-out microtubules in the growth

cone. RAB-11-positive endosomes are responsible for localizing the microtubule nucleation complex γ-TuRC to this ncMTOC. The MTOC tracks the extending growth cone by kinesin-1/UNC-116-mediated endosome movements on distal plus-end-out microtubules and dynein-mediated endosome clustering near MTOC. Critically, perturbation of the function or localization of the MTOC causes reversed microtubule polarity in dendrites. These findings unveil the dendritic MTOC as a critical organelle for establishing axon-dendrite polarity.

P1052/B176

The Role of Microtubule Pushing Forces in Aster Translocation.

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The cytoplasm of a eukaryotic cell is a complex and dynamic microenvironment that requires proper organization to insure cell function and homeostasis. The microtubule (MT) cytoskeleton plays a critical role in establishing this organization. During interphase, MTs serve as both a supportive scaffold for organelles and as an arborized system of tracks for intracellular transport. During mitosis, the position of the interphase MT aster determines the eventual location of the spindle apparatus and ultimately the cytokinetic furrow. Asters reliably move to the center of a cell, even in large blastomeres hundreds of microns in diameter and in unnaturally imposed cell geometries like squares and rectangles. These movements require the generation of positioning forces and in this context there are thought to be three primary forces: (i) MT pushing forces generated by the interaction between growing MT plus ends and the cell cortex, (ii) cortical pulling forces produced by MT dependent motors (dynein) anchored at the cell cortex, and (iii) cytoplasmic pulling forces generated by dynein-mediated transport of vesicular cargo through the viscous cytoplasm. How these different forces are integrated to position asters within cells of varied sizes, geometries, and in different systems remains an open question. To address it, we have developed the use of microfluidic devices and photolabile hydrogels to capture and release artificial microtubule organizing centers (aMTOCs) in geometrically defined hydrogel microenvironments. Combined with cell-free extracts, we use this system to dissect the integrated contributions of these three models for force generation in MT asters. We found that asters are able to find the geometric center of artificial microenvironments with similar kinetics to controls even when dynein motor activity was inhibited. To further test predictions of the different models, hydrogel barriers in the shape of a “V” with different interior angles were generated with aMTOCs positioned at their vertexes prior to MT nucleation. After the onset of MT nucleation, aMTOCs move away from the barriers. The velocity of their translocation was dependent on the “V”, with smaller interior angles producing faster speeds and increased distance away from the vertexes. Suggesting an underlying mechanism that relies more on proximity to a barrier and less on the MT center of mass. In summary, these data suggests that MT pushing forces contribute to aster translocation and positioning in *Xenopus laevis* egg extract and suggest that such forces could work over sufficient length scales to ensure aster positioning in vivo.

P1053/B177

Exploring the Mechanics of Microtubule Aster Positioning Using Photopatterned Hydrogel Enclosures.

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During interphase of the cell cycle, the microtubule (MT) cytoskeleton forms a radial aster with a center that is typically coincident with that of the cell. This centration insures proper positioning of the nucleus and sets up the antero-retrograde axis required for normal vesicular transport and cell function. Ultimately, it dictates the position of the mitotic spindle and the cytokinetic furrow, thus setting up a symmetric or asymmetric cell cleavage and proper partitioning of cytoplasmic contents between the two daughter cells. Despite intensive study, the molecular mechanisms and biomechanics that underlie aster positioning in different cell geometries and types remain elusive. Several aster centering models have been postulated, one class that relies on pushing forces generated by growing MTs as they impinge against the cell cortex and another that relies on cytoplasmic dynein-dependent pulling against either the cortex or elements in the cytoplasm. Progress in differentiating between these models and determining the exact underlying mechanism has been difficult, in part due to limitations with current experimental approaches. To overcome these limitations, we have developed a novel experimental approach that combines microfluidic-based technologies and photocatalyzable hydrogels to create defined geometries around MT asters in cell-free *Xenopus laevis* egg extract. Using this approach, we found that MT asters indeed center in cylindrical enclosures over a range of diameters and that the asters exhibit a tendency to rotate. Over the range of cylinder diameters tested, MTs exhibited buckling, an observation inconsistent with pulling force models. Interestingly, changing the geometry of the enclosures also had an effect on rotation, with angular velocity being inversely correlated with device diameter (i.e. faster rotation in smaller cylinders). Based on these observations, we believe that forces generated by growing MTs as they push against enclosure walls are responsible for the observed rotation, which implies that pushing forces can be exerted over length scales much larger than expected for unsupported, single MTs. We postulate that the aster MT array might be made more able to bear a compressive load by crosslinking or by motor-dependent packing of vesicular cargoes proximal to their centers.

P1054/B178

Self-organization of Microtubules in Cell-sized Droplets.

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We combine droplet microfluidics and cell-free biological systems to examine the effect of confinement and nucleation on the assembly of microtubule (MT) networks. Central to the spindle assembly is the spatial organization of MTs, a long tubular structure formed through the polymerization of tubulin dimmers. Such organization is regulated by RanGTP, a GTPase associated with chromosomal activities and acting as part of a major nucleation pathway for MTs. RanGTP has been explored using Xenopus egg extracts, a model cell-free system for probing spindle assembly. Most extract-based assays were performed in a test tube where cell-sized confinement was missing. Therefore, we asked whether confinement can affect the MT networks. We used droplet microfluidics for encapsulating extract-based assays by generating monodisperse, extract-in-oil droplets. By varying droplet diameters and encapsulated Ran concentrations, we demonstrate that these two physical factors regulate the assembly of MT networks. Together, the two factors yield MT networks with various steady-state

architectures. Our results highlight the prominent role of MT nucleation in the self-organization of MTs in cell confinement and might have direct implications in nucleation-controlled soft material processing.

Sensory and Signaling Functions of Cilia

P1055/B180

Cilia Protein Regulation of Actin Dynamics.

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Primary cilia are highly conserved multifunctional cell organelles that extend from almost every eukaryotic cell type. These microtubule-based appendages are vital for the development and homeostasis of different organs and tissues and play a role in the transduction of intra- and extracellular cues. Defects in the development of primary cilia can lead to a range of genetic disorders, collectively termed ciliopathies. The archetypical ciliopathy is the Bardet-Biedl syndrome (BBS), patients of which display many different phenotypes such as retinopathy, kidney dysfunction, obesity and polydactyly. Causative for this are mutations in the BBS genes, the proteins of which are predominantly involved in the intraflagellar trafficking inside the cilium. Recent data indicate that many ciliary proteins are also found at non-ciliary locations within the cell, for example at the cell membrane. This association might explain why loss of 'ciliary' function results in cell migration defects, possibly via regulation of the actin or microtubule cytoskeleton. To investigate the alternative functions of classically defined 'cilia' proteins, we performed a large-scale yeast-two-hybrid screen with the BBS proteins. One of the identified interactions was with the actin bundling protein Fascin. Further biochemical analyses validated a direct interaction between Fascin and BBS6 (MKKS). We could also show that this interaction may occur in an actin-dependent manner, since mutations in the actin-binding site of Fascin decreased the interaction. Live imaging of *Bbs* knockout fibroblasts also revealed a potential dysregulation of both Fascin and actin dynamics in the absence of Bbs, which is currently under further investigation. In addition, further analysis has revealed a transient interaction between Fascin and Inversin, a protein known to regulate migration and interact with several BBS proteins, suggesting potential two-way cooperativity between Fascin and cilia proteins. Our findings provide more insight into the molecular mechanisms of ciliary signaling and protein networks in the context of the Bardet-Biedl syndrome, which could further explain how cilia proteins affect cell migration and actin dynamics in a cilia-independent manner.

P1056/B181

Developmental and Odour Induced Localization of Olfactory Receptor Co-receptor Orco into the Olfactory Cilia of *Drosophila Melanogaster*.

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Organisms have an amazing ability to sense and distinguish between a plethora of odours present in the environment. The ability to do so is encoded in the olfactory sensory neurons (OSNs) in the form of olfactory receptors (ORs). ORs localize in the olfactory cilia, which are microtubule-based bipartite structures extending from the dendrites of the olfactory sensory neurons (OSNs). In *Drosophila melanogaster*, the ORs associate with a co-receptor called Orco, forming a functional ORx/Orco complex

which is essential for the localization of the OR to the distal segment of the olfactory cilia [1]. However, the mechanisms by which the ORx/Orco complex localizes into the olfactory cilia is not well elucidated. To understand the dynamics of ORx/Orco complex localization into the olfactory cilia, we monitored the enrichment of Orco in the olfactory cilia during development. We find that a significant amount of Orco enters the outer segments of olfactory cilia in a biphasic manner just after the emergence of the adult fly from the pupa. Further, to understand the molecular players involved in the process of Orco localization, we looked at the role of Kinesin-2, a motor protein attributed in trafficking of ciliary cargoes [2]. We find that Orco localization is reduced in the olfactory cilia upon OSN specific knock down of Kinesin-2. Suggesting the requirement of Kineisn-2 in Orco transport. On looking at the dynamics of Kinesin-2 during the enrichment of Orco into the olfactory cilia, we find that there is no change in the levels and the rates of Kinesin-2 localization in the ciliary outer segment during this period. We also find that the levels of Orco localization in the ciliary outer segment increase upon odour stimulation. Altogether, our results suggest that developmental cues and odour stimulation regulate the Orco/ORx complex entry into the cilia, and Kinesin-2 motor could play an essential role in this process. **References:** 1. Benton R, Sachse S, Michnick SW, Vosshall LB (2006) Atypical Membrane Topology and Heteromeric Function of *Drosophila* Odorant Receptors in Vivo. PLoS Biol 4(2): e20. 2. Verhey KJ, Dishinger J, Kee HL (2011) Kinesin motors and primary cilia. Biochemical Society Transactions 39 (5), 1120-1125.

P1057/B182

Primary Cilia Sense and Respond to Tubule Flow Changes Following Renal Ischemia-reperfusion Injury.

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Primary cilia are small microtubule based appendages that are present on nearly every mammalian cell type. In the kidney, primary cilia are found extending into the lumen of the tubule off the epithelium where they have classically been thought to function as mechanosensors that detect changes in fluid flow. In response to changes in tubular flow rates, it is proposed that primary cilia regulate signals to the nucleus to alter gene transcription thereby influencing the biology and outcome of that cell. Our preliminary data using live intravital microscopy in mice receiving intravenous injection of a fluorescently tagged dextran indicate that unilateral ischemia-reperfusion (IR) injury to the kidney results in disrupted tubular flow in a majority of tubules. Further, our preliminary data indicate that dextran is taken up by proximal tubule epithelial cells experiencing normal flow and that the number of cells taking up the dextran is reduced following IR injury. The dextran can remain in cells initially experiencing tubular flow for up to two weeks following the initial injection, functioning as a long-term marker of cells that at one period had tubular flow. Our preliminary data using intravital imaging also indicate that some tubules that initially lost flow can regain it at a later time point after IR injury. In the future, we propose to utilize a combination of flow cytometry, single cell RNA sequencing, and longitudinal intravital imaging to determine what effect loss of tubular flow has on epithelial gene expression, primary cilia structure and function, and cell division. We will also determine transcriptional changes that occur in tubules that at one period had flow, lost it following IR injury, and then regained it at later periods. Lastly, we are also performing these studies in murine models in which primary cilia structure or function are disrupted to determine the importance of primary cilia for normal tubular injury and repair processes. This will allow us to elucidate novel pathways involved in the renal injury and repair pathways and their relation to the primary cilium.

P1058/B183

Sensory Cilia Are a Sorting Nexus for Extracellular Vesicles.

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Extracellular vesicles (EVs) are emerging as a new dimension in intercellular communication. Compared to conventional intercellular signaling molecules such as hormones or cytokines, EVs allow donor cells to reprogram target cells through exchanging a package of proteins, lipids and genetic material. Understanding the cellular processes of EV biology is essential to shed light on the physiological and pathological functions of these vesicles as well as on therapeutic applications. We developed *C.elegans* as system to study EV functions and biogenesis mechanisms in a living animal (Wang et al 2014; Wang et al 2015). Cilia both shed and absorb EVs. Cilia are microtubule-based projections on the cell surface and serve as ubiquitous sensory organelles. Mutations in genes required for cilia formation or function result in wide spectrum of genetic diseases termed ciliopathies. By studying how cilia make and receive EVs, we aim to uncover fundamental principles of how cells communicate in health and disease. A subset of *C. elegans* sensory cilia make EVs abundantly. The ciliary receptor polycystin-1/LOV-1 and ciliary TRP channel polycystin-2/PKD-2 are evolutionarily conserved EV cargoes in both *C. elegans* and human. Polycystin-containing EVs are released into environment directly and function in animal-to-animal communication. Using fluorescently-tagged EV cargoes such as PKD-2::GFP combined with superresolution Airyscan confocal imaging, we identified two sites of ciliary EV production: at the cilia base and at the cilia tip, and that different mechanisms drive EV base and tip shedding. The biogenesis of the ciliary tip EVs depends on intraflagellar transport. The ciliary tip is a nexus of EV cargo sorting: different types of EV cargoes are sorted to EVs independent of each other. Strikingly, ciliary tip EVs are made in response to sensory activity of the cilia. In contrast, ciliary base EV biogenesis does not depend on ciliary trafficking and activity. Our work reveals how polycystin-containing EVs are made by cilia, adding an important component to understanding the fundamental biology of EVs and their potential relationship to ciliopathies.

P1059/B184

APEX2-proximity Proteomics Reveals That Trypanosome Flagellum Tip Is a Cell Signaling Focus.

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Trypanosoma brucei is the protozoan parasite causative of sleeping sickness, a vector borne disease that disrupts the sleep-awake cycle and is fatal if left untreated. The flagellum of *T. brucei* plays critical roles in parasite biology, transmission and pathogenesis. As the flagellum emerges from the cytoplasm, it is surrounded by membrane and is laterally connected to the cell body, forming a flagellum attachment zone (FAZ) along almost its entire length, with a short distal tip that extends beyond the cell body. An emerging concept in flagellum biology is the idea that the organelle is organized into subdomains, each having specialized composition and function. A critical gap in knowledge is the proteome of each flagellum subdomain. We have therefore used APEX2-proximity proteomics to examine protein composition from flagellum subdomains in *T. brucei*. Four different flagellar proteins were APEX2-tagged: one from the axoneme, two from the flagellar membrane at the tip, and one from the flagellar membrane along the FAZ. As expected, the axoneme proximity proteome gives good overlap with known proteomes from purified axonemes, validating the APEX2 system in trypanosomes. The tip proteome is distinct from the axoneme proteome and comparing the relative abundance of proteins in

the tip and FAZ proteomes allowed us to identify proteins concentrated in the flagellum tip. Interestingly, the flagellum tip is enriched with signaling proteins, including several proteins involved in cAMP and Ca++ signal transduction. We have independently assessed the localization of several proteins to the flagellum tip, validating our tip proteome. We have also tested roles in cell viability and are currently testing roles in social motility, a behavior known to depend on cAMP signaling at the flagellum tip. Finally, we have been able to combine APEX2-proximity labeling with TiO_2-dependent phosphopeptide enrichment. We have preliminary data showing that some of the proteins identified in the tip are indeed phosphorylated. Considering that phosphorylation is a hallmark of cell signaling, we are interested in studying the cAMP-dependent phosphorylation at the flagellum tip, and its role in social motility. In summary, we have demonstrated APEX2-proximity proteomics is effective in trypanosomes and can be used to resolve proteome composition of flagellum subdomains.

P1060/B185

Intraflagellar Transport Is Deeply Integrated in Hedgehog Signaling.

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Cilia monitor the extracellular environment through ciliary-localized receptors allowing the cell to coordinate its physiology with surrounding cells. Hedgehog signaling, the best studied ciliary pathway, plays fundamental roles during development and many of the developmental defects due to ciliary dysfunction are caused by abnormal hedgehog signaling. Key components of the pathway are enriched in the cilium and their localization changes in response to pathway activation. In the off-state, patched-1 (Ptch1) accumulates in cilia and prevents smoothened (Smo) ciliary accumulation and activation. Upon binding of ligand, Ptch1 exits the cilium, Smo is derepressed and accumulates in the cilium. This subsequently activates downstream signaling, leading to Gli transcription factors accumulation at the ciliary tip before their modification and translocation to the nucleus where they modulate expression of target genes. Ciliary trafficking of hedgehog components is poorly understood. Their movement is partly facilitated by intraflagellar transport (IFT) and perturbing IFT disrupts hedgehog signaling. IFT, which is critical for ciliary assembly and maintenance, involves motor driven transport of IFT particles consisting of ~30 proteins organized from IFT-A, IFT-B and BBSome subcomplexes. The IFT particle provides binding sites for diverse ciliary cargoes. We previously showed that Ift25 and Ift27, subunits of IFT-B, are not required for ciliary assembly. Instead, they work with Lztfl1 and the BBSome to regulate hedgehog signaling and maintain proper levels of Smo and Ptch1 in cilia during signaling. In this work, we explore how hedgehog controls the dynamics of Smo's ciliary localization by controlling Smo's interaction with the IFT machinery.

P1061/B186

***Ex Vivo* Live-cell Imaging of Outer Segment Traffic in Mouse Photoreceptors.**

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Photoreceptor cells are polarized cells that traffic a large volume of protein to their modified primary cilia, the outer segment. While the ciliary outer segment membrane is contiguous with the plasma membrane surrounding the cell body there is a diffusional barrier between the two compartments that allows for proteins to be specifically retained in or excluded from the outer segment. Previous work has shown that many outer segment-resident proteins (e.g. rhodopsin, peripherin, R9AP) contain a protein motif that targets them specifically to the outer segment. Interestingly, when these targeting motifs are

mutated the protein still reaches the outer segment but also non-specifically "spills" into the membrane surrounding the rest of the cell. The subtle differences in the kinetics, trafficking routes and delivery of outer segment-targeted and non-targeted membrane proteins remains unknown. To examine this, we generated a protein reporter to monitor the live dynamics of intracellular trafficking in *ex vivo* mouse retinas. A luminal, pH-sensitive fluorescent probe (superecliptic pHluorin, SEP) was fused to a single-pass transmembrane domain followed by cytosolic mCherry and rhodopsin's C-terminus with or without its targeting signal. Both constructs were then in *vivo* electroporated into wild-type mouse rods to confirm their expression and localization patterns. We then performed live time-lapse imaging from fresh, *ex vivo* sections of electroporated mouse retinas to compare the dynamics, kinetics and trafficking route of outer segment-targeted vs non-targeted membrane proteins. In the future, we will expand this system to other targeting signals used to reach the outer segment as well as explore how retinal degeneration impacts outer segment trafficking overall.

P1062/B187

The Role of Matrix Stiffness on Ciliogenesis.

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Primary cilia are small immotile microtubule based appendages that are present on most cell types. They act as cellular sensors that receive diverse signals from the extracellular environment. Upon ciliogenesis, the cilium can adjust its length in response to chemical and/or mechanical stimuli, and in turn, can enhance signal detection. Conversely, cilia shortening or resorption decreases responsiveness to signals, providing a mechanism of sensory signal adaptation. The modulus of elasticity, or stiffness, of the extracellular matrix (ECM) has been demonstrated to be a major determinant of ciliogenic activity. In addition to ECM stiffness, the actin cytoskeleton is also a dominant inducer of ciliogenesis. Recent studies revealed that actin destabilisation increases ciliogenesis and cilia length. The yes-associated protein (YAP), and transcriptional co-activator of the PDX-motif (TAZ) proteins are intracellular mediators of mechanical cues and ECM rigidity. Increased intracellular stresses caused by culture on stiff substrates leads to nuclear accumulation of YAP/TAZ, whereas spatial confinement or a soft substrate promotes YAP/TAZ cytoplasmic retention. More recently, cytoplasmic retention of YAP/TAZ was shown to correlate with ciliogenesis in cells treated with actin destabilisers, supporting a direct connection between the actin network, ciliogenesis and mechanosensing. However, the relationship between tissue mechanics, actin network and primary cilia are still not well understood. Therefore, we examined the effect of matrix stiffness on the primary cilia structure and function and its role in mechanosignalling. The role of the ECM in cellular mechanosensing is commonly studied in *vitro* using engineered hydrogels of tunable stiffness. Here, we used gelatin methacrylol with a stiffness gradient from 5 to 45 kPa. Murine articular chondrocytes were seeded on hydrogels for 24 hours, followed by low serum media for 48 hours, and fixed in 4% paraformaldehyde. Cells were then immunofluorescently labelled with ARL13B (ciliary protein), YAP (mechanosensitive protein) and phalloidin (F-actin). Our results showed an increased number of actin stress fibres with an increased substrate stiffness. There was also a positive relationship between increased YAP nuclear retention in the cells with increased substrate stiffness. However, our results showed that cilia length and frequency had an inverse linear relationship with an increased substrate stiffness. The length shortened from 3 µm to 1.7 µm and the percentage of ciliation decreased from 35% to 25% across the stiffness gradient hydrogel. We show that substrate stiffness

alone can fine tune cilia length, and therefore, lead to increased sensitivity in mechanosensing in the cells.

P1063/B188

A Cytoplasmic Protein Kinase in *Chlamydomonas* Links an Adhesion Receptor-activated Ciliary Signal to Cyclic AMP-mediated Cellular Responses, Including Mobilization of More Adhesion Receptors to the Cilia.

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Use of cilia to respond to environmental cues arose early in evolution. cAMP-mediated ciliary signaling in vertebrates is crucial for development and homeostasis, and disruption of cilia is associated with multiple human disorders. Many cilium-based signalling pathways rely on regulated redistribution of membrane signaling proteins between the organelles and the rest of the cell, yet we know little about the mechanisms that couple ligand binding on the cilium to a cellular responses. During sexual reproduction in the bi-ciliated green alga*, Chlamydomonas*, interactions between adhesion receptors on the cilia of gametes of opposite mating type (SAG1 on mating type *plus and* SAD1 on *minus*) trigger the sexually quiescent cells to become activated for fusion. cAMP produced by a ciliary adenylyl cyclase activated by SAD1-SAG1 interactions triggers cells to release their extracellular matrix, erect fusogenic membrane protuberances between the cilia; and, in a positive feedback loop, rapidly (< 5 min) mobilize a pool of SAG1 from the plasma membrane to the ciliary membrane to maintain and enhance adhesion. Here, we report identification of a gamete-specific protein kinase (GSPK), present primarily in the cytoplasm, that couples SAG1-based ciliary adhesion to responses in the cell body. Gametes bearing a mutant allele of *GSPK* retain ciliary adhesiveness, but are incapable of gamete fusion. Immunoblotting studies with cells expressing HA-tagged GSPK show that the protein is gamete-specific and present primarily in the cell body. *gspk* mutant gametes are capable of initial SAG1-dependent ciliary adhesion, but fail to mobilize SAG1 from the plasma membrane and thus fail to maintain ciliary adhesiveness. Failure to recruit SAG1 is a reflection of the overall inability of the mutant gametes to become activated by adhesion: fusion organelles fail to form and the extracellular matrix remains intact. On the other hand, *gspk* mutant gametes are fully capable of being activated by a cell-permeable form of cAMP. Moreover, cAMP assays show that ciliary adhesion in the mutant gametes fails to trigger the large, sustained increases in cAMP characteristic of adhering wild type gametes. Our results support the model that GSPK is essential for coupling the adhesion-induced increase in ciliary cAMP to the increase in cAMP in the cytoplasm required for gamete activation, and raise the possibility that cAMP-mediated responses to ciliary signalling in multicellular organisms might also depend on cytoplasmic production of cAMP.
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P1064/B189

A Centriole-less Pericentriolar Material Serves as the Base of *C.elegans* Sensory Cilia.

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Cilia are microtubule-based cellular appendages that assemble on basal bodies, which in some cell types are derived from centrioles. Perturbation of cilia related genes leads to a large scope of diseases, ciliopathies, such as retinal degeneration and polycystic kidney disease. While the assembly of cilia by basal bodies is shared among unicellular organisms and animal cells, in the nematode *Caenorhabditis*

elegans basal bodies are necessary for ciliogenesis, but are degraded upon cilia maturation. This raises the question of how ciliary microtubules are maintained in the absence of the basal bodies from which they are templated. We used CRISPR-generated endogenously tagged alleles to assess the localization of centrosomal proteins. Surprisingly, although the base of cilia lacks an association with centriole proteins or the PCM protein SPD-2/CEP192, SPD-5, ZYG-9/XMAP-215, TAC-1/TACC3 and the conserved microtubule nucleating γ-tubulin ring complex (γ-TuRC) localize to this region. Superresolution microscopy revealed distinct subdomains of these proteins, with a subset localizing more proximal to the ciliary axoneme and others localizing more distally to a region nucleating the assembly of dynamic microtubules into the cell body, confirming that the base of cilia is a microtubule organizing center (MTOC). Using tissue-specific degradation, we tested the role of γ-TuRC and SPD-5 at the base of cilia at different time points in development. Degradation of SPD-5 and the γ-TuRC component GIP-1/GCP3 after cilia maturation did not grossly impact cilia structure or function and γ-TuRC was not required for SPD-5 localization to the base of cilia. However, degradation of SPD-5 during ciliogenesis perturbed cilia structure, suggesting that SPD-5 is required to maintain the axoneme in the absence of a canonical basal body. Unlike at the centrosome, the localization and regulation of SPD-5 appears to be independent of mitotic kinases, as CDK-1/CDK1 and PLK-1/PLK1 do not localize to the base of cilia. Further degradation and imaging studies will reveal a more complete map of the base of cilia and explain the molecular control of both ciliogenesis and MTOC function. Additionally, we hypothesize that the base of cilia represents a pure MTOC structure stripped from the centrosome and so these studies are likely to reveal novel regulation of MTOC function at the centrosome.

P1065/B190

Location-dependent Noncanonical Elemental Composition and Cellular Architecture of the Statolith and Underlying Cells in the Early-diverging Metazoans, *Beroe Ovata* and *Mnemiopsis Leidyi*.

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Ctenophores, the largest animals that move primarily by means of beating cilia (the comb plates), are thought to have diverged from all other multicellular animals at least 350 mya, and the ultimate origin of the entire group is probably set much deeper in time. In this context, the remarkable observation by Tamm's group recently of the unusual organization, mechanisms of construction and cellular origin of the statolith has considerable import, because it describes for the first time an alternative, extremely ancient solution to a common problem: the organization and structure of a gravity-sensing organ. Here, we explore further the exceptional characteristics of these cells. A common characteristic of bio-deposited gravity sensing structures across animals is that they are calcareous: containing calcium. We sought to determine the composition and organization of the lithocyte concretions in order to better appreciate how this ancient group would respond to changing pH in the world's oceans. We confirmed, using DNA staining (Hoechst 33342, 1 µg/mL), that the lithocytes are MOSTLY nucleated; not all labeled with the stain. Newly forming lithocytes were not associated with Hoechst-positive staining. We used energy dispersive spectroscopy (EDS) and electron microprobe analysis (EMPA) to compare and contrast the composition of ctenophore lithocytes from the Woods Hole (WH) region and the northern Gulf of Mexico (nGOM). We found several distinct features common to all of the ctenophores, with distinct differences by location. 1) lithocytes from both species, whether examined by EDS or EMPA, lacked Ca or Mg and did not display any divalent cations. 2) the primary composition of the positive ion was that of Na, and in some cases, potassium, although sodium was the most common positive univalent ion present. 3) Oxygen, which was detectable by EDS but no by our EMPA system, was always present. 4)

Sulfur was always present. 5) Cl was much more evident in the nGOM samples. 6) Crystal structure was very uniform in the WH samples. There was variable structure in the nGOM crystal arrays in dried down samples, but 7) intact lithocytes, while very refractile, were distinctly non-birefringent. The relative contribution of the different elements varied somewhat by location, probably indicative of the local nutrient available to the animals, which could reflect a very adaptive and opportunistic metabolism. We interpret these unexpected results to be indicative of an ancient metabolism carried down to the present day. We acknowledge the generous help of Dr. Oldenbourg, and Mr. L. Kerr, Marine Biological Laboratory. Supported by Auburn University, Office of the Vice President for Research.

P1066/B191

Probing Photoreceptor Outer Segment Status Using Genetically-encoded Secreted an nexin V.

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The Jensen laboratory is interested in how photoreceptor outer segments develop and are maintained. Vertebrate photoreceptors are specialized light sensing neurons that have unique cellular morphology and die in retinal degeneration diseases, including macular degeneration, retinitis pigmentosa and many other inherited retinal degenerations. The photoreceptor outer segment is a highly modified primary cilium where photons of light are captured and translated into changes in membrane potential that alter synaptic neurotransmitter release. The rod outer segment (ROS) has the typical ciliary axoneme but, in addition, alongside the axoneme, are hundreds of densely packed, stacked, descrete intramembranous discs that concentrate membrane-associated phototransduction proteins. Outer segments are continuously regenerated or renewed throughout the life of the animal through the combined processes of proximal outer segment growth and distal outer segment shedding. The shed outer segment material is ingested and digested by the neighboring retinal pigmented epithelium (RPE). We seek to define the principles and molecular mechanisms that control proximal growth, distal shedding and total length of outer segments during development (net outer segment elongation) and during homeostasis in the adult. It was reported that ROS tips are labeled with an nexin V (A5) in isolated live mouse retina preps in which the RPE was peeled away from the outer segment tips (Ruggiero et al., *PNAS* 109:8145-8148, 2012). A5 is used to probe live cells for surface binding to phosphatidylserine, which has been flipped from the inner leaflet to the outer leaflet, that serves as an 'eat-me' signal to phagocytic cells. To help reveal molecular mechanisms and the dynamic process of ROS shedding, we sought to develop a method to conditionally and temporally express a V5-epitope tagged secreted A5 (secA5 ^{V5}) in live zebrafish retina. We generated a transgene construct in which a tetracycline response element (TRE) controls secA5^{V5} expression. To observe mosaic gene expression and to produce sources of secA5^{V5}, we injected this construct into three different transgenic lines we made that express a tetracycline-controlled transactivator (tTA) under cell specific promoters: RPE specific expression (*rpe65a* promoter), cone specific expression (*UV opsin* promoter), rod specific expression (*rhodopsin* promoter). Using anti-V5 antibodies and confocal microscopy we examined outer segments and found no elevated V5 labeling at outer segment tips, although cone outer segments were weakly labeled along their entire length. We propose that either V5-binding is poor in intact live retinas in our experiments or that the V5 binding previously reported is a product of mechanical force following removal of the RPE.

P1067/B192

TTBK2 and Primary Cilia Are Essential for the Connectivity and Survival of Cerebellar Purkinje Neurons.

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Primary cilia are essential microtubule-based signaling organelles found on nearly all mammalian cells, including glia and neurons. While cilia play a well-known role in neural development, their function within the adult nervous system remains largely unknown. Tau Tubulin Kinase 2 (TTBK2) is a critical regulator of ciliogenesis. Additionally, mutations in *Ttbk2* are linked to a dominantly inherited neurodegenerative disease, spinocerebellar ataxia type 11 (SCA11). Recent work from our lab found that SCA11-associated alleles of *Ttbk2* dominantly interfere with cilia formation and function, highlighting a new role for this protein in cilium stability. Using mouse models, we aim to further define the requirements for TTBK2 and cilia in adult neural function, and to examine the links between ciliary dysfunction and neurodegenerative diseases like SCA11. To address this goal, we used conditional alleles of *Ttbk2* and *Ift88* crossed to an inducible, ubiquitously expressed Cre (*UBC-CreER*) to genetically remove cilia throughout the brain under temporal control. *Ttbk2*$^{f/f}$*;UBC-CreER* (*Ttbk2*$^{c.mut}$) animals exhibit rapid loss of cilia throughout the cerebellum upon Cre induction following completion of cerebellar development (P21). These animals develop motor coordination deficits as well as loss of synapses on Purkinje cell (PC) dendrites by four months of age. Similarly, *Ift88*$^{c.mut}$ have cilia defects throughout the cerebellum, and show the same loss of PC synaptic connections. By six months of age we observe a loss of PCs in both *Ttbk2*$^{c.mut}$ and *Ift88*$^{c.mut}$ animals, reminiscent of models of SCA subtypes. Additionally, we have found that these changes result predominantly from cell-autonomous defects to the PCs: We have found that *Ttbk2*^{PCP2} animals, in which recombination occurs specifically in PCs, exhibit nearly identical phenotypes to those of *Ttbk2*$^{c.mut}$ animals. Taken together, our data demonstrate that primary cilia in the adult brain are essential to maintain the connectivity and viability of a key population of neurons. Our findings also point to ciliary dysfunction as a potential cellular mechanism underlying some types of neurodegeneration.

P1068/B193

RAB-28 Regulates Ciliary Extracellular Vesicle Formation.

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Extracellular vesicles (EVs) are tiny communication devices that cells release to influence cell, tissue, and organism behavior. One locale that cells release EVs from is the cilium- adding signal releaser to the previously ascribed role as a signal receiver. Given the cilium's profound importance as a signaling organelle, two questions that need to be studied are- 1. How do cilia make and release EVs? 2. What is the function of ciliary EVs? the EV releasing neurons (EVNs) of *C. elegans* provide an excellent in *vivo* system to study ciliary EVs. *C. elegans* release evolutionarily conserved EV cargoes such as the polycystin PKD-2 into the environment. Environmentally released EVs are bioactive and regulate animal-to-animal communication. Transcriptional profiling of the EVNs provided a list of candidates for a role in EV biology including *rab-28* - a ciliary GTPase associated with autosomal cone-rod dystrophy in humans. Previous studies showed that *C.elegans rab-28* acted in neuronal cilia to regulate sensory pore size possibly via neuron signaling to glia. The unidentified nature of the neuronal signal to glia, and EVN enrichment of *rab-28* prompted us to examine whether RAB-28 functions in ciliated cells via regulating

EVs. We find that *C. elegans* RAB-28 is a negative regulator of EV shedding using a combination of genetics, live imaging, and transmission electron microscopy. In wild type animals, EVs are predominantly observed within sensory organs in a lumenal space surrounding the base of the cilium with fewer EVs observed in the part of the lumen that surrounds the distal regions of cilia. *rab-28* mutants however, accumulate an excessive amount of EVs in the lumenal space surrounding distal regions of cilia. Interestingly, *bbs-8* mutants phenocopy *rab-28* EV and sensory pore expansion phenotypes revealing a functional relationship between the two. We also identify that lipidated RAB-28GTP is first trafficked to the periciliary membrane, and then to the ciliary membrane. This sequential trafficking of RAB-28 depends on the BBSome and PDE6D respectively. Thus, our studies also indicate a transport relationship between BBS-8 and RAB-28. We conclude that RAB-28 and BBS-8 are in *vivo* negative regulators of ciliary EV shedding within sensory organs and that one function of ciliary EVs may be to mediate neuron to glia communication within a sensory organ.

P1069/B194

Septin-mediated Rhoa Activation Regulates Ciliary Assembly and Trafficking.

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Primary cilia are non-motile protrusion of the plasma membrane found on nearly every vertebrate cell. The organelle senses and transduces diverse signals to regulate cell physiology, development and organ homeostasis. Mutations disrupting ciliary structure and function cause a wide range of human disorders collectively known as ciliopathies that affect nearly all organs. In order to perform the critical functions, both the lipid and protein compositions of ciliary membranes are distinct from those of the contiguous cytosol and plasma membrane. This specialized composition of the cilium is mediated by the transition zone at the junction between the basal body and the axoneme. The transition zone functions as a ciliary gate to control the protein entry and exit from the cilium and the possible trafficking of essential ciliary components. One of the septin family genes, SEPTIN2 was previously identified to be crucial for ciliogenesis where it mediates the localization of transition zone components to the ciliary base. Septins are filamentous GTPases that act as macromolecular scaffolds and as regulators of membrane protein diffusion that have been implicated in diverse biological processes including cytokinesis, cell morphogenesis and motility, and ciliogenesis. The goal of this study was to further examine the requirement of septins and its molecular mechanism in the context of ciliogenesis. Research in our lab has shown that SEPTIN9 is essential for the proper formation of primary cilia in RPE cells and regulates RhoA signalling at the base of cilia by binding and activating a specific guanine nucleotide exchange factor for Rho, the septin-associated Rho-GEF. Given that the exocyst complex, which functions in docking secretory vesicles to sites of polarized secretion, is a downstream effector of RhoGTPases, we have shown that potential ciliary proteins are trafficked to the base of cilia by the exocyst complex to maintain the ciliary composition. Thus, our work has provided a mechanistic explanation of how SEPTIN9, through activating RhoGTPase and its downstream effector exocyst, mediate ciliary assembly and trafficking.

P1070/B195

Volumetric Electron Microscopy Reveals New Details of Primary Cilia Organization and Potential Function in the Brain.

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Signaling through primary cilia contributes to both development and function of the brain, but we currently have few insights into the roles and spatial organization of cilia on different cell types. Neuropeptides and neurotransmitters emerge from, and pass through, the dense network of axons, dendrites and glial processes known as the neuropil to activate cilia-mediated signaling pathways. For practical reasons, most strategies implemented to study primary cilia visually isolate cilia from this complex network. To elucidate the context in which primary cilia reside in brain tissue, we analyzed cilia on more than 200 mouse visual cortex cells in volumetric transmitted electron microscopy (TEM) data. In addition, we characterized a cilium from the nucleus accumbens in an isotopic focused ion beam-scanning electron microscopy (FIB-SEM) volume. We investigated both the structural features of neuronal and astrocytic cilia and the cellular and extracellular environment surrounding them. In the neuropil, cilia were proximal to glial processes, axons and dendrites. In addition, many neuronal and astrocytic cilia were adjacent to at least one synapse - close enough to theoretically detect spillover signals if they contain relevant receptors. On pyramidal neurons, cilia emerged at a distance from the axon initial segment and either traveled along the edge of the soma for part of their length, or projected directly into the neuropil. In contrast, most astrocyte cilia in the visual cortex were recessed in a deep ciliary pocket. We observed endo/exo-cytic events on the plasma membrane adjacent to the cilium, as expected. However, we also found areas where the plasma membrane hosted extensive endoplasmic reticulum-plasma membrane contact sites adjacent to where the cilium abutted the plasma membrane. Tracking cilia into the neuropil using volumetric TEM also revealed structural differences between neuronal and astrocytic cilia. Together, FIB-SEM and volumetric TEM of primary cilia are providing a new nanoscale understanding of cilia structure and cellular organization. The resolution and large volume of these data allow us, for the first time, to quantify differences between cilia of different cell types, measure changes along the lengths of cilia, and generate hypotheses about the origin of ciliary signals in the brain.

P1071/B196

For the LOV of CRISPR: Generating Endogenous L*OV-1* Alleles to Study Polycystin Mediating Signaling in Cilia and EVs in *C. Elegans*.

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Human autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited ciliopathies and common cause of end-stage renal failure. The polycystins PKD1 and PKD2 are mutated in ~95% of ADPKD cases with PKD1 mutations occurring most often at around 85% of cases. C. elegans provided one of the first links between cilia and human disease. The C. elegans polycystins LOV-1 and PKD-2 localize to cilia and function in a sensory capacity to regulate male mating behaviors. Remarkably, polycystin localization to cilia and extracellular vesicles (EVs) is conserved, suggesting an ancient function in these signaling organelles. EVs are tiny (100-200 nm) membrane-bound vesicles that deliver lipids, nucleic acids, and proteins to neighboring cells. Cilia are important sites for EV release and interaction. In humans, evidence suggests that polycystin-1 and polycystin-2 form a complex as a 1:3

heterotetramer whose function remains an enigma 25 years after their identification. The worm is a powerful model for understanding the fundamental biology of the polycystins, cilia, and ciliary EVs. Moreover, PKD-2 location on ciliary EVs is essential for EV bioactivity. To determine the function of the polycystins in cilia and ciliary EVs, we use CRISPR/cas9 genome editing and fluorescent microscopic techniques to engineer tagged-versions and disease causing mutations. LOV-1 molecular functions have not been well studied because of its size (355 kDa). To tackle this problem, we tagged endogenous LOV-1 with an N-terminal scarlet tag and C-terminal NeonGreen tag. Our data show that LOV-1 highly expressed in male-specific EV-releasing neurons and localizes to cilia and ciliary EVs. LOV-1 and PKD-2 overlap in localization, consistent with co-trafficking and function as a complex. Preliminary data suggests that LOV-1 is cleaved, presumably at the conserved GPS proteolytic site, and the N-terminal and C-terminal cleavage products may traffic together or independent of each other. To understand the function of this evolutionary conserved cleavage site we are now generating mutants to see how removal of this site affect LOV-1 trafficking, localization to cilia/ciliary EVs, and function in male behaviors and ciliary EV signaling. Our findings will reveal in vivo functions of the polycystins in cilia and ciliary EVs, which may inform understanding of the human polycystins in normal and ADPKD states.

P1072/B197

Unraveling the Role of the Jade Protein Family in Cilia Biology, Cell Cycle Regulation, and Renal Cell Carcinoma.

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ecent groundbreaking studies could demonstrate that primary cilia play a pivotal role in the pathogenesis of cystic kidney diseases and cancer biology. As sensory organelles cilia transmit signals from the environment into the cell and modulate multiple signaling pathways. Over the last decades genetic studies have identified numerous disease-causing mutations in a multitude of genes that can cause cystic kidney disease and related ciliopathies. Here, we focus on the cilia associated E3 ubiquitin ligase JADE1 and its family members JADE2 and JADE3. JADE1 targets the Wnt downstream effector ß-catenin for proteasomal degradation and has been found to interact with various ciliopathy-associated proteins including pVHL, NPH proteins and polycystin-1. pVHL is the major tumor suppressor in the kidney, while mutations affecting NPH proteins or polycystin-1 can cause different types of (poly)cystic kidney diseases. Thus, JADE1 might modulate the pathogenesis of both, renal cell carcinoma and cystic kidney disease. To understand the in *vivo* function of Jade1 we generated a whole body knockout mouse model. These mice do not show any obvious phenotype. This is in accordance with our hypothesis that due to the high similarity of the Jade protein family members and their expression in the kidney, there could be compensatory mechanisms in place. Further experimental evidence reveals shared functions of Jade proteins and supports this notion: Co-immunoprecipitation experiments demonstrate that JADE2 and JADE3 interact with previously described interactors of JADE1, including NPHP1, NPHP4, and pVHL. Moreover, we could show that JADE1/2/3 are co-precipitating with each other respectively. To further elucidate the function of Jade2 and Jade3 and their potential to compensate for Jade1 in *vivo* we also successfully generated whole body knockout mouse models for Jade2 and Jade3. Furthermore, we generated and validated Jade1/2/3 deficient murine inner medulla collecting duct cell lines. These cell lines show alterations in the proliferation rate while the knockout of one individual Jade family member affected expression levels of the remaining Jade proteins. First unbiased MS/MS analyses revealed only

subtle changes in protein expression. We will now use these cell lines, together with cell lines expressing endogenously GFP-tagged Jade proteins, to analyze the function of Jade proteins as transcription factors using RNA-Seq and ChIP-Seq studies combined with proteomic profiling. In summary, our data suggest that the high structural similarity of the Jade protein family members is recapitulated in their ability to perform overlapping functions. Moreover, we demonstrate novel functions of Jade proteins which points out a fundamental role of Jade proteins in kidney epithelial cells.

Spindle Assembly 1

P1073/B199

Opposing Active Forces Provide Mechanical and Functional Robustness in the Mammalian Spindle.

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During cell division, microtubules and motors generate force to build the spindle. At metaphase, the spindle reaches an active, energy-consuming steady state in which dynein provides contractile force via microtubule minus-end clustering, and the kinesin Eg5 drives extensile microtubule sliding. These motors antagonize each other: dynein depletion leads to unfocused and turbulent spindles, while Eg5 depletion causes spindles to contract into monopoles. When both are absent, however, the spindle can establish its normal bipolar architecture. Here we ask what role these opposing forces play, and whether their functional advantages outweigh their energetic cost. We generate doubly-inhibited spindles by knocking out NuMA, thereby preventing dynein-based end-clustering, and inhibiting Eg5 with STLC. We find that these spindles attain a typical bipolar shape, fully align their chromosomes, and proceed through anaphase. However, despite their shape and mitotic progression, doubly inhibited spindles are less mechanically robust: under external force, they exhibit dramatic shape instabilities. Further, they give rise to more lagging chromosomes, suggesting that their altered mechanics compromise their function. Thus, opposing contractile and extensile forces in the spindle are critical to ensure that chromosome segregation occurs accurately despite internal and external stresses. Our findings provide insight into energy consumption as a strategy to promote robustness, an engineering design principle found in diverse biological contexts such as metabolism, phosphoregulation, and membrane fission and fusion.

P1074/B200

***In Vitro* Sliding of Microtubules by XCTK2 Molecular Motors: Towards an Understanding of Microtubule Flux in the Spindle.**

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We present experiments and theory on a system of stabilized microtubules driven by the molecular motor protein XCTK2. Through photobleaching experiments, we demonstrate that in this system microtubules are aligned along the long direction of the system and travel through the gel at a velocity independent of the local average polarity. We show that this result is most naturally understood in the framework of an active gel theory that goes beyond pairwise microtubule interactions and treats the gel as highly cross-linked. Our theory bridges the length scales from the microscopic mechanical behavior of motor-filament interactions to the large scale behavior of the active gel and generalizes to describe

different kinds of cytoskeletal assemblies. Importantly, it might explain the physical origin of microtubule sliding in similar in *vivo*system like the spindle.

P1075/B201

Kinesin-5 Cross-linking and Sliding Ensures the Stability and Function of Nascent Bipolar Spindles.

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One of the most important properties of the mitotic spindle is its bipolar structure. But how a nascent bipolar spindle is established and maintains a stable structure capable of efficient chromosome capture and generating kinetochore tension is poorly understood. The budding yeast mitotic spindle is an outstanding model system for this study due to its minimal microtubule architecture, allowing structural investigation by electron microscopy and the wealth of tools for live cell fluorescence imaging that we used probe new questions regarding the formation and properties of nascent spindles and the proteins that influence these properties. Here, we use live cell fluorescence microscopy to show that the monopolar (duplicated but unseparated spindle poles) to bipolar spindle transition is one of the fastest steps in spindle assembly and is irreversible. Using Kinesin-5 mutants that target its ability to slide apart microtubules we determine that microtubule crosslinking and not sliding by Kinesin-5 is the main driver of this transition from monopolar spindles to nascent bipolar spindles. By fluorescently tagging the microtubule associated proteins that have been implicated in bipolar spindle formation we demonstrate that Cin8 (Kinesin-5) is the first to load onto the monopolar spindle prior to bipolar spindle formation even when its microtubule sliding capacity is inhibited. This is consistent with the electron microscopy tomography analysis we have undertaken that reveal that the monopolar spindle's microtubule architecture is comprised principally of short high angle microtubules unsuited for ensemble Kinesin-5 sliding. Following this fast and irreversible transition we show using live cell imaging that nascent spindles require Kinesin-5 sliding to ensure a stable equilibrium length that allows for further spindle elongation prior to anaphase onset. If Kinesin-5 sliding is inhibited it gives rise to shorter nascent spindles that exhibit larger length fluctuations that persist to anaphase onset. Finally, our preliminary results show that when Kinesin-5 sliding is inhibited mitotic spindles present defects in kinetochore clustering. Such Kinesin related defects may reveal that how one of the earliest steps in spindle assembly may have long range effects in chromosome segregation.

P1076/B202

Mammalian Kinetochore-fibers Set Their Length and Dynamics Independently of Spindle Poles.

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How do cells use the same nanometer-scale components to build different micron-scale structures? During mitosis, cells reorganize tubulin building blocks into various microtubule structures to build the spindle. In mammalian cells, bundles of dynamic microtubules called kinetochore-fibers (k-fibers) extend from chromosomes to poles, driving chromosome segregation. While the length of the spindle is important for its function, and many proteins that modulate spindle length are known, the basic principles underlying length establishment remain poorly understood. To define the role of spindle architecture in this process, I generate barrel-shaped spindles using a dynein inhibitor, where k-fiber minus-ends no longer focus into a pole. I find that these spindles' k-fibers have an unchanged mean length, but more varied lengths within each cell, and yet that each k-fiber's length is maintained over

time. This suggests that pole-independent mechanisms establish and also maintain the k-fiber's length scale, and that pole activities contribute to homogenizing lengths. Further, my preliminary work shows that k-fibers flux poleward and can regrow after ablation in these spindles. Thus, the pole is not essential in regulating and coordinating plus- and minus-end dynamics, contrary to proposed models. Furthermore, laser ablation of barrel k-fibers reveals that k-fiber microtubules modulate their dynamics in a length-dependent manner. Together, my findings suggest that length is an emergent property of k-fibers, independent of global spindle architecture or of dynein-based contractile forces. Thus, using physical and molecular approaches, I aim to dissect how k-fibers 'know' their length, a micron-scale property, to actively regulate their local dynamics at the nanometer-scale.

P1077/B203

The Partitioning of the Genome into Chromosomes Affects Mitotic Spindle Metaphase Behavior but Not Spindle Assembly.

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A fundamental question in biology is how the partitioning of the genome into a set number of chromosomes impacts the fidelity of mitosis. Here we test the hypothesis that the number of chromosomes determines the structure and function of the mitotic spindle, using an approach that integrates computational models of mitosis in fission and budding yeast with experiments. Using the interspecies modeling approach, where the organisms package a similarly sized genome into 3 (fission) and 16 (budding) chromosomes, we demonstrate that changes to chromosome number disrupt mitotic fidelity and identify interdependent relationships between chromosome number, microtubule dynamics, and kinetochore-microtubule binding properties. One key difference between models is that budding yeast requires slower microtubule dynamics. In addition, we present preliminary experimental data from yeast cells in which chromosomes have been fused to decrease the total number to understand how the spindle can adapt to these changes. Spindle assembly is similar, but spindles are longer with larger length fluctuations during metaphase, in chromosome fusion strains when compared to the wild type. These results from modeling and experiment suggest that the number of chromosomes do affect the structure and function of the mitotic spindle, especially in metaphase.

P1078/B204

Probing Force Balance in the S. Pombe Mitotic Spindle by Laser Ablation.

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The S. pombe mitotic spindle forms as a single bundle of parallel and antiparallel microtubules. During elongation, the spindle extends via antiparallel microtubule sliding by molecular motors. These extensile forces from inside the spindle resist compressive forces from the nucleus and pushing by astral microtubules. We probe the source of this force balance via laser ablation of spindles at various stages of mitosis. We find that spindle pole bodies collapse toward each other following ablation, but that spindle geometry is often rescued, in some cases allowing spindles to resume elongation and chromosome segregation to complete. While this basic behavior in response to ablation has been previously observed [1,2], many open questions remain as to the timing, mechanics, and molecular requirements of these phenomena. The earlier work suggested that the spindle collapse after ablation was due to viscoelastic relaxation of the chromosomes and nuclear envelope. However, here we simultaneously observe the behavior of microtubules and the nuclear envelope during spindle collapse

and find that it is inconsistent with compressive pushing from the envelope as the sole source of spindle collapse. These data suggest that some other force is bringing the ablated halves of the spindle together. We also quantify the time scales of both the relaxation and rescue responses and begin to probe the molecular requirements affecting both spindle collapse and rescue. [1] A. Khodjakov, S. La Terra, F. Chang. Curr. Biol., 2004, 14:15, 1330-1340.[2] I. Tolic-Nørrelykke, L. Sacconi, G. Thon, F. Pavone. Curr. Biol. 2004, 14:13, 1181-1186.

P1079/B205

Analysis of Kinetochore Movements Suggests Deterministic Nature of Mitotic Spindle Assembly.

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The goal of cell division is to segregate chromosomes equally into two daughter cells. To achieve this goal, each chromosome must attach to the opposite poles of the 'mitotic spindle', a self-assembling molecular machine built from microtubules. Attachments are formed via capture of dynamic microtubules by the 'kinetochores', a pair of macromolecular assemblies residing on the opposite sides of the chromosome's centromere. A major postulate of the popular "search & capture" (S&C) hypothesis is that capture is stochastic and therefore kinetochores become attached to microtubules independently at different times. Here we test this postulate by following 3-D movements of spindle poles and all kinetochores in human cells at 5-s temporal resolution. Machine-learning analysis of kinetochore trajectories reveals three distinct chromosome behaviors during prometaphase: 1) chromosomes that continuously remain near the equator of forming spindle, 2) chromosomes that initiate movement towards a spindle pole but rapidly return to the equator, and 3) chromosomes that become associated with a spindle pole for a prolonged time. Relative frequencies of these three behaviors change to various extents when the activities of dynein and CenpE, two molecular motors residing at the kinetochore, are inhibited individually or together. Further, we identify characteristic signatures in the chromosome trajectories, such as the amount of active movement, timing of centromere rotations, changes in the distance between sister kinetochores, and patterns of chromosome oscillation. By utilizing these quantitative signatures to cluster chromosome trajectories, we reconstruct the sequence of molecular events that gives rise to each of the three chromosome behaviors. Our analyses suggest that stable equatorial position is achieved via two distinct pathways. In the first pathway, chromosomes initiate transient lateral interactions with spindle microtubules during the early stages of spindle assembly but do not undergo extended active movements within the spindle. In the second, initiation of lateral interactions with microtubules is delayed but the active movements at the later stages of spindle assembly are prominent. Our analyses also suggest that transition from lateral interactions with microtubules to stable attachments of sister kinetochores to the opposite spindle poles (biorientation) occurs in synchrony at the stage determined by spindle geometry. Thus, chromosome behavior during prometaphase is not consistent with the predictions of S&C hypothesis. This mechanism of spindle assembly appears to be deterministic rather than stochastic.

P1080/B206

Towards Measuring In-vivo Ndc80c Binding Kinetics from Flim-fret Fluctuations.

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The coupling of the kinetochore to microtubules drives many aspects of chromosome motion during cell division. Despite recent advances in understanding the composition and assembly of the kinetochore, the mechanism of force generation at the kinetochore remains poorly understood. The NDC80complex (NDC80c) is believed to be the primary coupler between microtubules and the kinetochore. The two most widely discussed models of forces production are the biased diffusion model, in which the N80c is proposed to transiently and dynamically associate with microtubules, and a conformational wave model, in which the NDC80c is proposed to engage in stabile, cooperative interactions with microtubules. Our group has previously developed a FLIM-FRET based method to precisely measure the fraction of NDC80c at individual kinetochores in U2OS cells bound to microtubules. Here, we first investigate the role of Hec1 N-terminal tail kinetochore microtubule coupling. We find that mimicking phosphorylation of the Hec1 N-terminal tail significantly alters the fraction of bound NDC80c. Second, we propose an extension of this methodology to measure the dynamics and cooperativity of NDC80c binding and unbinding to microtubules at kinetochores via analysis of fluctuations in the FLIM-FRET signal. We still present theory, simulations and preliminary pilot experiments using diffusing dyes and DNA hairpins arguing that the proposed approach is promising. The same methodology could be applied to study the kinetics of other biochemical reactions in-vivo, on timescales from seconds to microsecond. Third, we explore the impact of microtubule-kinetochore binding on k-fiber dynamics. We will present preliminary results from photoactivation experiments examining the impact of microtubule-kinetochore coupling on k-fiber structure and tubulin turnover. We aim to use these photoactivation measurements to test models of k-fiber dynamics.

P1081/B207

Understanding the Forces That Move Chromosomes and Position the Spindle.

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During cell division, the mitotic spindle, a dynamic, microtubule-based molecular machine, attaches to and exerts forces on chromosomes to first align them along the spindle equator and then move them apart. The position and orientation of the spindle determine the cell's cleavage plane, which ultimately determine how cellular contents are split between the two daughter cells. Therefore, dynamic and precise of regulation of the forces within the spindle that align and separate chromosomes and the positioning forces on the spindle itself is of crucial importance to mitosis. Previous studies have identified many molecular players involved in chromosome separation, however it has been difficult to investigate mechanical forces over the course of mitosis in live cells, mainly due to a lack of tools. We used a magnetic tweezer system to apply forces of varying magnitude and timescale on the spindle with intracellular magnetic beads. Preliminary results show that for short pushes, the spindle recovers its original position, however for longer pushes, the equilibrium position of the spindle appears to move and the original position is not recovered. By combining molecular perturbations with physical measurements, we plan to dissect the mechanistic details of chromosome motion and spindle positioning.

P1082/B208

Measuring the Force Generated by the Fission Yeast Mitotic Spindle.

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Measuring forces within cells is an important step in understanding how cellular processes function mechanically. An open question in mitosis is how much force the mitotic spindle exerts to segregate chromosomes. In fission yeast, the spindle can be regarded as a simple bundle of overlapping microtubules that elongates during anaphase to push the nuclei and chromosomes apart. We have discovered conditions under which the majority of cells to fail in anaphase. In these cells, chromosomes cannot fully segregate and the spindle continues to elongate and gradually adopts a bent shape rather than its normal straight shape. Ultimately these spindles fail abruptly, by either breaking in half or by one pole disassociating from the chromosomes. We hypothesize that spindles exert pushing forces to elongate and bend their microtubules into this shape. Using simple beam theory, we estimate that the spindle exerts nanoNewton forces to bend itself, and that similar forces are needed to break the spindle [1][2]. This magnitude of force is consistent with the predicted action of 100's of motor proteins which each provides picoNewton forces [3]. Future directions include determining what factors, such as motor proteins and crosslinkers, contribute to force production. Our findings in fission yeast provide quantitative measurements and will ultimately shed light on the contribution of mitotic components in force production for more complex spindles such as in animal cells.

P1083/B209

Modeling the Dynamics of Centrosome Movement.

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Mitosis is the process of cell division, resulting in two genetically identical daughter cells. This process is regulated by the mitotic spindle, composed of a complex network of microtubules (MTs) and motor proteins. These structures generate pushing and pulling forces allowing the formation of a bipolar spindle, with each pole organized around a single centrosome. Disruptions in force generating activities through protein depletions or alterations to centrosome number corrupt spindle structure and affect the fate of the cell in mitosis. Centrosome amplification, a common characteristic of cancer cells, promotes multipolar spindle formation and multipolar division, which results in daughter cells with decreased viability. As such, cancer cells actively cluster extra centrosomes to form a functional bipolar spindle and undergo a bipolar division. To define forces that are required for efficient spindle pole clustering, we are developing a novel biophysical model of spindle formation in cells with extra centrosomes by solving force balance equations to determine the position of the centrosomes in time. We consider stochastic force generators at the cell cortex and between interpolar microtubules. To inform the model and test explicit predictions that it generates, we are using live-cell imaging and image processing to track centrosome movement in cells.

P1084/B210

Kinetochore-fibers Locally and Slowly Dissipate Force to Maintain Robust Mammalian Spindle Structure.

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At cell division, the mammalian kinetochore binds many spindle microtubules that make up the kinetochore-fiber. To segregate chromosomes, the kinetochore-fiber must be dynamic, and must both generate and respond to force. How the kinetochore-fiber responds to force remains poorly understood: it cannot be reconstituted in vitro, and exerting forces on it in vivo remains challenging. Based on Nicklas' classic work, we developed a microneedle manipulation assay to pull on mammalian kinetochore-fibers close to kinetochores and probe how force regulates their dynamics and structure. We show that force lengthens kinetochore-fibers by persistently, and reversibly, favoring their growth versus shrinkage, rather than by increasing growth rate. Using photomarking, we demonstrate that lengthening occurs by preventing depolymerization at both microtubule plus- and minus-ends, and not by microtubules sliding within the bundle. Finally, even under forces sufficient to rupture kinetochore-fibers we do not observe detachment from kinetochores or poles. Together, this work suggests strategies for spindle structural homeostasis under force: kinetochore-fibers locally dissipating force at both ends, behaving mechanically as a single unit, and having strong spindle connections at force dissipation sites. These simple engineering principles may be helpful for understanding a broad range of dynamic, force-generating cellular machines.

P1085/B211

Cortical Pulling Force Drives Pronuclear Migration and Rotation, and Spindle Positioning and Oscillation.

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In the *C. elegans* early embryo, the pronuclei complex migrates to the cell center and rotates such that the mitotic spindle forms along the anterior-posterior axis. Subsequently, the spindle undergoes transverse oscillations as it moves toward the posterior. This asymmetric spindle positioning is crucial for asymmetric cell division and proper development. The contributions of pushing, cortical pulling, and cytoplasmic pulling forces to those dynamic centrosome positioning events are not fully understood. To study these processes, we constructed a novel laser ablation system capable of creating nearly arbitrary 3D cuts of astral microtubules at any desirable timing. We used this system to dissect the relative contribution of pushing and pulling forces throughout pronuclei and spindle motions. Our results suggest that all of these motions are dominated by net pulling. We used microinjected fluorescent nanodiamonds to track cytoplasmic fluid flow, which indicates that cortical pulling forces dominate over cytoplasmic pulling at all stages. We used computer simulations and mathematical modeling to interpret our experimental data. Taken together, our results strongly argue that cortical pulling drives pronuclear migration and rotation, metaphase spindle positioning, asymmetric spindle positioning and all aspects of spindle oscillations.

P1086/B212

Hierarchical Regulation of Spindle Scaling during Development.

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Cells need to regulate the size and shape of their organelles for proper function. For example, the mitotic spindle adapts its size to changes in cell size over several orders of magnitude, but we lack a mechanistic understanding of how this is achieved. We have recently shown that the upper spindle size is set by the limited activity of microtubule nucleators around chromosomes, even when components are unlimited. For small spindles, however, it has been shown that microtubule dynamics correlate with spindle size during development. Thus, the mechanisms of cell-size dependent spindle scaling are still unclear due to the lack of experimental data on the underlying microtubule processes in a system that covers the full regime of spindle size scaling. Here, we combine quantitative microscopy, laser ablation, and modelling in zebrafish embryos and encapsulated *Xenopus* egg extract to reveal the relative contributions of microtubule length dynamics and nucleation to cell-size dependent spindle scaling. We find a hierarchical regulation of spindle scaling. In large spindles, microtubule dynamics and turnover are independent of spindle size—implying a scale-invariant microtubule length. Below a threshold cell size, microtubule polymerization dynamics and turnover correlate with spindle size. However, the change in microtubule dynamics alone is not sufficient to account for the observed spindle scaling. Instead, we find that microtubule nucleation is the main biophysical process that scales spindle size across all cell sizes, although microtubule dynamics still fine tune spindle size in small cells. We hypothesize that this hierarchical regulation of spindle scaling is an inherent consequence of building spindles from microtubules that are smaller than the size of the spindle. The gradual transition from a microtubule nucleation-based to a microtubule dynamics-based mechanism of spindle assembly implies a change in spindle architecture, and may ensure faithful chromosome segregation by spindles of all sizes.

P1087/B213

Microneedle Manipulation of the Mammalian Spindle Reveals Specialized, Short-lived Reinforcement Near Chromosomes.

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The spindle generates force to segregate chromosomes at cell division. In mammalian cells, kinetochore-fibers (k-fibers) connect chromosomes to the spindle. Yet, how the dynamic spindle anchors k-fibers in space and time remains poorly understood since we lack tools to directly challenge this anchorage. Based on Nicklas' pioneering work, we develop a microneedle manipulation assay to exert local forces on the mammalian spindle with spatiotemporal control. Pulling on k-fibers in the lateral axis away from the spindle reveals the spindle's ability to retain local architecture in response to forces applied for seconds. Upon pulling, sister, but not neighbor, k-fibers remain tightly coupled, and the spindle globally shortens rather than k-fibers detaching from poles or chromosomes. The deformed shape of k-fibers reveals that they can freely pivot around poles, but not kinetochores, remaining unbent within the first 2µm near kinetochores. This local reinforcement near kinetochores has a lifetime of ~20 s under force, and requires the microtubule crosslinker PRC1. Together, these observations suggest strong, short-lived, and specialized reinforcement of the k-fiber in the spindle center. Such reinforcement could help the

spindle robustly retain its local structure at dynamic sites of force generation, while allowing spindle remodeling on longer timescales.

P1088/B214

The Mitotic Crosslinking Protein PRC1 Acts as a Viscous Dashpot Against Relative Microtubule Sliding.

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Cells use cytoskeletal networks to perform complex physical tasks throughout diverse processes such as the assembly of the mitotic spindle during cell division. These dynamic networks consist of microtubules that are organized, bundled, and transported by motor and non-motor proteins that produce 'active' pushing and 'passive' frictional forces to achieve proper assembly and perform mechanical work. How these mesoscale forces are regulated at the micron-scale by ensembles of nanometer-sized proteins has been unclear. We are addressing this knowledge gap by directly measuring force production across microtubule bundles using optical tweezers and simultaneously observing by single molecule fluorescence microscopy the localization of key proteins that regulate network stability. Here, we demonstrate that ensembles of PRC1, an essential non-motor crosslinking protein needed both to assemble bridging fibers during metaphase and build the central spindle in anaphase, are viscous frictional elements whose resistance to motor-driven microtubule sliding scales linearly with velocity and local protein concentration. Our direct experimental measurements and computational simulations describe how PRC1 molecules can both diffuse within the overlapping microtubule network and track a partially reflective barrier at microtubule plus-ends in order to act as a viscous dashpot against microtubule sliding motions. This mechanical property can then help to establish stable rates of both chromosome and pole separation. These results set the groundwork for understanding higher-order microtubule networks as "machines" that use simple rules to modulate their force production and control the spatiotemporal organization of the dynamic cytoskeleton.

P1089/B215

A Minimal Spindle-Midzone Protein Module Differentially Regulates Single Microtubules and Crosslinked Microtubule Arrays.

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A remarkable feature of the microtubule cytoskeleton is the co-existence of distinct sub-populations with different stabilities in the cellular cytoplasm. A striking example is the spindle midzone, an array of antiparallel microtubule bundles that encodes positional cues for accurate cytokinesis in dividing cells. At the cell center, these stable bundles exist alongside dynamic microtubules that are thought to contribute to midzone organization. How do stable bundles and dynamic single microtubules coexist at the center of the dividing cell? Here we reconstitute a minimal system composed of the antiparallel microtubule crosslinker PRC1, and its binding partners Kif4A and CLASP1, midzone-associated proteins that suppress and promote microtubule growth respectively. We find that the activity of these proteins on dynamic microtubules results in a system with stable bundles and elongating single microtubules. An alysis of the protein-protein interactions reveals that two parameters are sufficient to explain the differential regulation of single and bundled microtubules: (i) higher microtubule affinity of CLASP1 over Kif4A and (ii) higher PRC1 affinity of Kif4A over CLASP1. Together with cell biological analyses, our findings provide new insights into how antagonistic regulators of microtubule dynamics can

differentially regulate proximal subsets of microtubules for midzone assembly. More broadly, our findings illuminate the design principles that can confer different stabilities to microtubule sub-populations that coexist at the same subcellular site.

P1090/B216

The Mechanical Integrity of the Mammalian K-fiber and Its Molecular Origin.

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During mitosis, the mitotic spindle, a self-constructed microtubule-based machine, segregates chromosomes into two eventual daughter nuclei. Microtubule bundles that align and anchor chromosomes, called kinetochore-fibers (k-fibers), are vital for the structural and functional integrity of this spindle, yet the composition and dynamics of k-fibers are still not well-understood. To probe k-fibers in mammalian PtK2 cells, we perform both high-resolution single-molecule speckle fluorescence imaging and mechanical perturbation by targeted laser ablation. In searching for the identities of microtubule crosslinking proteins that mechanically support k-fiber integrity, we biochemically and genetically perturb candidate molecules in spindles, ablate k-fibers, and subsequently quantify the physical response of the k-fiber. While speckle microscopy experiments in unperturbed spindles demonstrate that k-fibers act as mechanically cohesive units that are held together along their lengths, laser ablation of k-fibers often results in the splaying of the remaining stubs. Together, these results suggest that forces holding k-fibers together are tuned to be of similar magnitude as other cellular forces. Following ablation, it has previously been shown that stub minus-ends are typically detected and pulled poleward, repairing the spindle. In spindles with reduced NuMA, a molecule that recruits dynein to microtubule minus-ends and mediates the post-ablation poleward transport, splaying is reduced, suggesting that when splaying occurs, it is may be because k-fibers are torn apart during poleward transport. Additionally, pharmacological inhibition of kinesin-12 Kif15 microtubule binding increases the frequency of post-ablation splaying, while inhibition of its motor activity but not its microtubule binding does not affect splaying, suggesting that Kif15 may fortify and stabilize k-fibers. Interestingly, inhibition of Kif15 also results in dramatically delayed reincorporation of ablated k-fibers, although the process by which Kif15 impacts spindle repair remains unclear.

P1091/B217

Self-organization of Spindle-like Microtubule Structures.

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Microtubule self-organization is an essential physical process underlying several essential cellular functions, including cell division. In cell division, the dominant arrangement is the mitotic spindle, a football-shaped microtubule-based machine responsible for separating the chromosomes. We are interested in the underlying fundamental principles behind the self-organization of the spindle shape. Prior biological works have hypothesized that motor proteins control the proper formation of the spindle. Many of these motor proteins are also microtubule-crosslinkers, so it is unclear if the critical aspect is the motor activity or the crosslinking. In this study, we seek to address this question by examining the self-organization of microtubules using crosslinkers alone. We use a minimal system composed of tubulin, an antiparallel microtubule-crosslinking protein, and a crowding agent to explore the phase space of organizations as a function of tubulin and crosslinker concentration. We find that the

concentration of the antiparallel crosslinker, MAP65, has a significant effect on the organization and resulted in spindle-like arrangements at relatively low concentration without the need for motor activity. Surprisingly, the length of the microtubules only moderately affects the equilibrium phase. We characterize both the shape and dynamics of these spindle-like organizations. We find that they are birefringent homogeneous tactoids. The microtubules have slow mobility, but the crosslinkers have fast mobility within the tactoids. These structures represent a first step in the recapitulation of self-organized spindles of microtubules that can be used as initial structures for further biophysical and active matter studies relevant to the biological process of cell division.

P1092/B218

Using a Small Molecule Inhibitor of Spastin to Probe Nuclear Envelope Reformation.

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In order to divide and propagate, cells must distribute replicated chromosomes into nascent daughter cells. In metazoans this occurs by “open mitosis,” in which disassembly of the protective nuclear envelope is required for the attachment of spindle microtubules to chromosomes at kinetochores. Strong connections between spindle microtubules and kinetochores are necessary to evenly distribute replicated chromosomes during anaphase, but persistent connections can impede subsequent nuclear envelope reformation. Recent work has proposed that spastin, a microtubule-severing AAA+ ATPase, is responsible for severing microtubules at chromatin interfaces in late anaphase, allowing the nuclear envelope to fully encapsulate chromosomes within minutes of segregation. However, spastin also has well characterized roles in endosomal trafficking during interphase and intercellular bridge disassembly at the end of mitosis. Standard genetic perturbations, which can take days before effects are observed, are therefore unable to resolve spastin’s specific role in nuclear envelope reformation. To overcome this limitation, we used spastazoline, a recently developed small molecule inhibitor of spastin, to acutely inhibit spastin activity during nuclear envelope reformation in late anaphase. Using live cell imaging, we tracked spastin accumulation at the reforming nuclear envelope and measured changes in spastin foci dynamics after inhibition with spastazoline. On average, individual spastin foci persisted at the nuclear envelope approximately 1.5-fold longer in the presence of spastazoline, while the average number of spastin tracks detected per cell increased 3.4-fold. We go on to use multimodal imaging to examine spastin foci and microtubules in late anaphase, characterizing the effects of spastin inhibition and expanding spastin’s role in nuclear envelope reformation.

P1093/B219

Understanding the Role of an euploidy and Chromosomal Instability in Chronic Inflammation.

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While the link between inflammation and cancer has been noted for well over 150 years, the molecular mechanisms driving this relationship remain unclear. In inflammatory bowel disease (IBD), which is characterized by chronic inflammation of the gastrointestinal tract, cells with abnormal chromosome stoichiometries (aneuploid) arise frequently. The questions of how these altered cells arise and what role they play in driving evolution from chronically inflamed tissue to cancerous lesion have yet to be explored. Our study aims to characterize the extent of karyotypic heterogeneity and mechanisms that drive emergence of aneuploidy and chromosomal instability (CIN), the process which gives rise to aneuploidy, in IBD. Gene set enrichment analysis reveals increased expression of genes associated with

CIN in colonic biopsies of IBD patients. Novel methods for analysis of single cell RNA sequencing data are utilized to identify karyotype abnormalities and alterations in inflammatory pathways in individual epithelial cells from patients with IBD. The influence of chronic inflammation on the karyotype of intestinal epithelial cells is studied in colon organoids derived from a DSS-induced mouse model of colitis. Our findings have the potential to unveil the mechanisms of progression from chronic inflammation to cancer, opening the door for preventive strategies to target these malignancies before they occur.

P1094/B220

Understanding the Role of Tp53 in Mediating an euploid Cell Fate in Different Mammalian Cells and Patient Derived Organoids.

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Equal partitioning of replicated chromosomes to daughter cells is an essential phenomenon to maintain genome stability. Chromosome mis-segregation events and aneuploidy are prevalent in majority of mammalian tumors. The role of tumor suppressor TP53 and its downstream target CDKN1A have been implied in regulating numerical and structural chromosomal aberrations in certain tissue types. The multi-layered complexity arising from different ways to induce aneuploidy and the role of tissue architecture make it difficult to study aneuploid cell fate in a rigorous and standardized manner. In this study acute aneuploidy was generated by inhibiting an essential spindle assemble checkpoint regulator MPS1, which resulted in cells passing through mitosis with irregular kinetochore and microtubule attachments and higher number of mis-segregated chromosomes in daughter cells. Using biochemical and genetic approaches on a comprehensive panel of cell lines and organoids from human and mouse we investigated the differential regulation of aneuploid cells by the TP53 pathway. External factors like adherence properties of 2D monolayer cells show a dependence on TP53 pathway to limit the proliferation of aneuploid mammalian cells. Whereas suspension culture, neural progenitor cells and mammalian organoids from colon and breast show an increased tolerance to aneuploid cell proliferation and indicate the presence of other molecular pathways to regulate aneuploid cell fate. These results indicate how altering the TP53 response can allow certain tissue types to tolerate higher amounts of aneuploidy and elucidating a possible route to aneuploidy-mediated genomic instability in carcinogenesis.

P1095/B221

Quantitative an alysis of the Signaling Dynamics of the Spindle Assembly Checkpoint Utilizing Crispr-cas9-mediated Genome Editing.

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The spindle assembly checkpoint (SAC) is a signaling pathway that delays anaphase onset in the presence of unattached kinetochores in a mitotic cell. How the number of unattached kinetochores changes the SAC signaling output is not fully understood. This knowledge will help us comprehend how the SAC prevents premature anaphase onset in the presence of a decreasing number of unattached kinetochores during normal mitotic progression. To address this question under physiological conditions, we used CRISPR-Cas9-mediated genome editing to fuse mNeonGreen to 3 of the core SAC signaling proteins in HeLa cells (Bub1, BubR1, and Mad1) and utilized GSK923295 (a CENP-E inhibitor) or

nocodazole to induce small or large numbers of unattached kinetochores in mitotic cells. To evaluate the importance of the abundance of Bub1, BubR1, and Mad1 to the robustness of the SAC in the presence of various numbers of unattached kinetochores, we partially knocked down each protein with RNA interference against mNeonGreen-tagged alleles. We observed that BubR1 knock-down is the most impactful compared to Bub1 and Mad1 at similar knock-down efficiencies, leading to a significant reduction in the duration of the mitotic arrest in cells containing small (but not large) numbers of unattached kinetochores. As the recruitment of Bub1, BubR1, and Mad1 to unattached kinetochores is crucial for the conventional SAC signaling pathway, we then quantified their localization in the presence of various numbers of unattached kinetochores. In mitotic cells containing small numbers of unattached kinetochores, each unattached kinetochore recruits significantly higher amounts of Bub1, BubR1, and Mad1. Importantly, the fold increase in BubR1 and Mad1 recruitment is significantly higher than in Bub1 recruitment. Finally, since Bub1, BubR1, and Mad1 localize to MELT motifs of the SAC scaffold protein Knl1 (each putatively has 19 such motifs in human) at unattached kinetochores, we studied the importance of the number of MELT motifs in each molecule of Knl1. We replaced the endogenous Knl1 with recombinant versions containing either 6 or 3 MELT motifs to examine how the SAC strength is affected. Cells exhibit a drastic enhancement in their SAC signaling strength when there are 6 MELT motifs per Knl1 compared to 3, but again only in cells containing small numbers of unattached kinetochores. Together, these results implicate BubR1 and the number of MELT motifs in Knl1 as key determinants of the robustness of the SAC in the presence of small numbers of unattached kinetochores.

P1096/B222

Paradoxical Mitotic Exit Induced by a Small Molecule Inhibitor of APC/C^{Cdc20}.

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The an aphase Promoting Complex/Cyclosome (APC/C) is a mega-dalton ubiquitin ligase that initiates mitotic exit by targeting substrates for degradation. The APC/C is activated by Cdc20, which acts as a substrate receptor, and is inhibited by the mitotic checkpoint complex (MCC), which delays mitotic exit when the spindle assembly checkpoint (SAC) is activated. We previously identified apcin, a small molecule ligand of Cdc20, as an inhibitor of APC/C^{Cdc20}. Surprisingly, we found that apcin paradoxically accelerates substrate degradation and promotes mitotic exit in cells with high SAC activity. Biochemical studies indicate that apcin cooperates with p31^{comet} to relieve MCC-dependent inhibition of APC/C. Apcin's behavior as an antagonist of Cdc20 can thus result in either net inhibition of APC/C, delaying mitotic exit when SAC activity is low, or net activation of APC/C, promoting mitotic exit when SAC activity is high. Genetic experiments suggest that the dual behaviors of apcin arise from targeting a common binding site in Cdc20 that is required for both substrate ubiquitination as well as efficient APC/C inhibition by MCC. We therefore establish a new mechanism through which a small molecule, by targeting a single site on a dynamic protein interface, can lead to opposing biological effects depending on the regulatory context.

6

Cytokinesis 2

P1097/B223

Deciphering the Roles of Discoidin Proteins in Controlling Cell Mechanics.

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Processes such as cytokinesis, migration, and chemotaxis require robust cell shape changes. To complete these processes, cells need to integrate internal and external cues through a mechanoresponsive machinery that includes actin filaments, myosin II motors, actin crosslinkers, and other scaffolding proteins. Uncovering and understanding the broad underlying network of proteins that drive and control this machinery is a prime interest of our research in our lab. Using *Dictyostelium discoideum*, we identified through proteomics and genetics interactors of the actin crosslinker cortexillin I and the scaffolding protein IQGAP2 as two key nodes of the machinery. We uncovered a network of biochemical interactors that potentially contribute to the activity and integrity of the system. Among those, discoidin proteins I and II are the focus of my research. I aim to decipher the roles and functions of discoidin proteins in the context of cell mechanics. Discoidin proteins are sugar binding proteins containing a lectin domain and a discoidin domain. Though several previous studies have identified roles of discoidin in cell adhesion and development, the exact functions of discoidins remain elusive. We previously found that discoidin I interacts with myosin II and cortexillin I in *vivo*. Now, I have used CRISPR-Cas9 to generate knockouts of discoidin and am investigating the defects in these knockouts using various mechanical indicators such as growth rates, number of multinucleated cells, assembly of myosin II, and the accumulation of mechanoresponsive proteins in response to applied mechanical stress. Further results will be discussed in the poster.

P1098/B224

Mitosis Specific Dynein Adaptor Hook2, Regulates the Cell Cycle Progression and Its Mitotic Localisations Are Regulated by the Mitotic Kinase Plk1.

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In addition to intracellular microtubule-based retrograde transport, cytoplasmic dynein also regulates events like centrosome separation, chromosome alignment and separation, spindle orientation and focusing during mitosis. Association of dynein, with its activator dynactin, dramatically enhances the processivity of dynein motor. This interaction is mediated by various adaptor proteins which form a stable ternary complex containing dynein, dynactin and the adaptor. The adaptors also link the cargo vesicle to dynein, thereby facilitating their transport. Hook proteins are coiled-coil proteins that regulate dynein motility by enhancing the association between dynein and dynactin. Unlike lower organisms where there is a single Hook gene, mammals express three Hook paralogs having distinct cellular localisation and non-redundant functions. In this study, we report that human HOOK2, a centrosomal protein also localises to central spindles and midbody during anaphase-B and cytokinesis, respectively. Furthermore, we noted that these additional localisations of HOOK2 depend on translocation of HOOK2 from centrosomes to these transient structures and require the activity of mitotic kinase PLK1. While the dynein association of HOOK2 is regulated by its evolutionarily conserved N-terminal Hook domain, a

second site in C-terminal region is crucial for binding to dynactin subunit p150^{glued}. The interaction of HOOK2 with dynein and dynactin is enhanced during mitosis. At the onset of mitosis, HOOK2 regulates anchorage of the centrosome to the nucleus, and microtubule nucleation from the centrosome and its RNAi mediated depletion results in a mild increase in mitosis duration, defects in mitotic spindle positioning, chromosome misalignment during metaphase. Furthermore, cytokinesis failure leading to the formation of binucleated cells was also observed upon HOOK2 depletion. HOOK2 mediates the interaction between dynein and dynactin and regulates their localisation on central spindles during anaphase. Further, HOOK2 via its ability to regulate dynein/dynactin also promotes dynactin-dependent recruitment of centralspindlin complex component MKLP1 to spindle midzone, thereby controlling the cleavage furrow ingression required for completion of cytokinesis. Our study reveals a novel role of centrosomal protein HOOK2 as a mitosis specific dynein adaptor, thereby regulating cell cycle progression.

P1099/B225

Elucidating Mechanisms Regulating an illin Function for Cytokinesis.

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Cytokinesis describes the physical separation of a dividing cell into two daughter cells. This process is driven by the formation and constriction of a RhoA-dependent contractile ring that forms at the cell equator in anaphase. Cytokinesis must occur properly as failure can lead to aneuploidy and cell fate changes, which are hallmarks of cancer. Thus, multiple pathways coordinate to couple ring assembly and ingression with chromosome segregation. While the dogma is that the anaphase spindle provides cues to control RhoA activity for cytokinesis, we recently found that a chromatin-sensing pathway also influences the contractile ring. This chromatin pathway functions via active Ran, which forms an inverse gradient to importins. Human anillin, a key cytokinesis regulator, contains an NLS that binds to importins and is required for its cortical recruitment. An illin also has binding sites for active RhoA and microtubules, and we are studying how they are coordinated. Reducing active RhoA enriches anillin's localization to microtubules, while increasing microtubules via MCAK (kinesin-13, microtubule depolymerase) RNAi decreases anillin's cortical localization. Thus, RhoA and microtubules compete with each other. An illin's cortical affinity is also reduced when the NLS is mutated. To determine how microtubule binding is altered in the NLS mutant, we performed co-sedimentation assays and found that anillin's binding domains for microtubules and importin partially overlap. Moreover, importin weakly competes for anillin's microtubule binding in vitro. This suggests that the NLS mutant should have reduced localization to microtubules in cells, and its decrease in cortical recruitment may not be caused by the same phenomenon as in MCAK RNAi cells. In support of this, FRAP experiments revealed that the NLS mutation and MCAK RNAi both cause an increase in the immobile fraction, but only the NLS mutation causes an increased off-rate. This supports the model that importin-binding is required for the robust cortical recruitment of anillin, by feeding back to stabilize anillin's interaction with RhoA or the membrane.

P1100/B226

Determining the Role of TTLL12 and the Tubulin Code during Cell Division.

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Post-translational modifications of tubulin regulate both the stability of microtubules as well as the interactions with microtubule motors and other microtubule-associated proteins. The patterning of these modifications has been proposed to constitute a tubulin code that functions in a variety of cellular events. However, despite the importance of microtubules in mediating cell division, what role, if any, the tubulin code plays during mitosis remains unknown. To investigate the potential role of the tubulin code in regulating mitosis, we analyzed the levels of tubulin glutamylation and glycylation during different stages of mitotic cell division. Our data show that mitotic spindle microtubules are heavily poly-glutamylated during metaphase and early anaphase. Interestingly, glutamylation levels significantly decrease during late anaphase and telophase. Finally, we found no evidence that mitotic or central spindle microtubules are glycylated. Thus, our data reveal that the tubulin code undergoes dynamic changes during mitotic cell division, although the function of these changes remains to be determined. The tubulin tyrosine ligase-like (TTLL) family catalyzes either glycylation or glutamylation of tubulin tails, and recently, we have identified TTLL12 as a potential regulator of mitotic cell division. Therefore, to determine the involvement of TTLL12 in regulating cell division we created a HeLa TTLL12 knock out cell line. Importantly, TTLL12 KO cells have an increase in multinucleation, suggesting an increase in mitotic failure. Furthermore, our data indicate that TTLL12 may regulate early steps of mitosis, such as mitotic spindle formation and metaphase-to-anaphase transition. TTLL12 is a unique member of the TTLL family since its enzymatic function remains unclear. To identify if a tubulin post-translational modification is mediated by TTLL12 we next analyzed the effect of TTLL12 knock-out on the tubulin modification levels during metaphase. Interestingly, our data show that TTLL12 depletion leads to an increase in tubulin poly-glutamylation while having little effect on acetylation or tyrosination. Overall our data suggest TTLL12 does not directly modify tubulin but rather acts a negative regulator of poly-glutamylation, consequently, mediating dynamic changes in the tubulin code during mitotic cell division.

P1101/B227

Spikes of Intracellular Calcium Accompany Fission Yeast Cytokinesis.

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Cytokinesis is the final stage of cell division during which two daughter cells separate. In some animal embryos, cytoplasmic calcium concentration increases sharply at the cell division plane during cytokinesis. *However, it remained unclear whether this process is evolutionally conserved and important for cytokinesis. To* address these questions, we measured intracellular calcium concentration during cytokinesis of the fission yeast *Schizosacchromyces pombe* with quantitative fluorescence microscopy. We employed GCaMP, a genetically encoded fluorescence calcium reporter, in our study. Expressed from an integrated genomic locus, GCaMP distributed homogenously throughout the cytoplasm. Intracellular fluorescence remained low among most GCaMP expressing cells, although it exhibited a weak correlation with the cell morphogenesis. Hypo-osmotic stress, a well-known stimulus of intracellular calcium, triggered instant spikes of the GCaMP fluorescence, confirming that GCaMP reports the intracellular calcium concentration of fission yeast cells as well. Among the dividing cells, very few calcium spikes were found during mitosis. In contrast, during cytokinesis, two distinct spikes of

intracellular calcium were recorded in almost all (>90%) dividing cells. The first one was concomitant with the initiation of the cleavage furrow ingression, agreeing with what was found in the animal cells. The second spike accompanied the initiation of cell separation, but it appeared asymmetrically between the two daughter cells. A calcium chelator EGTA reduced the rate of ring closure and triggered post-cytokinesis cell lysis in a dosage-dependent manner, suggesting that calcium homeostasis may be important for the fission yeast cytokinesis. We conclude that intracellular calcium spike accompanying cytokinesis is an evolutionally conserved process and calcium may play an important role in both the furrow ingression as well as the cell separation.

P1102/B228

A Putative Trp Channel Pkd2p an tagonizes the Sin Pathway to Maintain Cell Integrity during Fission Yeast Cytokinesis.

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A Putative TRP Channel Pkd2p an tagonizes the SIN Pathway to Maintain Cell Integrity during Fission Yeast Cytokinesis Debatrayee Sinha, Madhurya Chetluru and Qian Chen* Fission yeast Pkd2p is a putative transient receptor potential (TRP) channel *and* the homologue of human polycystins. Our lab recently found that Pkd2p localizes to the cleavage furrow and is essential for the cell separation. However, it remains unclear how this ion channel contributes to cytokinesis. *Here we show that a key role of Pkd2p during cytokinesis is to maintain the cell integrity during cell separation by antagonizing activities of the SIN pathway. The* Septation Initiation Network (SIN) is an essential Hippo-like signaling cascade that regulates cytokinesis. We first identified strong positive genetic interactions between *pkd2* mutants and many SIN mutants. Even though the SIN pathway activity is essential for both the cell integrity and septation, during cytokinesis. Pkd2p only antagonizes its role in maintaining cell integrity. Depletion of Pkd2p in the SIN mutants prevented these cells from lysis. The activity of the SIN pathway can be directly measured by the asymmetric distribution of Cdc7p between two spindle pole bodies (SPB). In the *pkd2* mutant, this asymmetry is enhanced. Cdc7p-GFP molecules exited significantly sooner from the SPBs of the p*kd2* mutant, compared to the wild type. Additionally, Pkd2p may also suppress the cytokinetic localization of the SIN pathway protein Mob1p. In the *pkd2* mutant, the peak molecular numbers of Mob1p on the spindle pole bodies and the contractile ring increased by more than 10%, compared to wild-type cells. We concluded that one key role of Pkd2p in cytokinesis is to maintain the cell integrity by modulating the activity of the SIN pathway.

P1103/B229

Parallel between Retroviral Budding and Cytokinesis: a Novel Factor for MBR an choring at the Cell Surface.

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Cytokinesis is the last step of cell division, leading to the physical separation of daughter cells. In animal cells, a dense structure located at the centre of the intercellular bridge, called the Midbody (MB), recruits the proteins necessary for abscission. The abscission occurs successively on each side of the MB, releasing a Midbody Remnant (MBR). The MBR is inherited asymmetrically by one of the daughter cells, and then roams over the cell surface during several hours before being eventually internalized and degraded in lysosomal compartments. Distinct MBR release and accumulation behaviours have been

observed according to the cell type, suggesting post-cytokinetic roles for MBRs. Interestingly, it has been proposed that the MBR could act as a signalization platform and regulate cell fate. For instance, the MBR has been shown to contribute to establishing the dorso-ventral axis in *C.elegans* embryo, and promoting primary ciliogenesis in mammalian cells. Despite the new focus on the post-mitotic roles of the MBR, the molecular mechanisms underlying these functions are still unknown but recent evidence indicates that its interaction with the cell surface promotes cell proliferation. However, which proteins interact and retain the MBRs at the cell surface are still poorly understood. Using MS/MS proteomic analysis of pure MBRs from human HeLa cells, we identified a tethering factor that is enriched at the midbody both during cytokinesis and on post-cytokinetic MBRs. Using the CRISPR/Cas9 knock-out technology, we reveal that this tethering factor is recruited to the MB prior to abscission, and then promotes the anchoring of the MBR at the cell surface, thus preventing its release. Mechanistically, a mutant form of this protein is unable to localize properly at the MB, and cannot efficiently tether the MBR. Intriguingly, this protein also promotes virion attachment to the cell surface. This work sheds new light on how MBRs remain attached to the cell surface, and reveals a new and unexpected parallel between viral restriction and cytokinesis.

P1104/B230

Coacervates of the Chromosomal Passenger Complex (CPC) Promote Microtubule Nucleation to Generate Complex Microtubule Structures.

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The Chromosomal Passenger Complex (CPC) is a major regulator of mitosis. It is composed of kinase Aurora B and its regulatory subunits Survivin, Borealin, and INCENP. The CPC interacts with complex microtubule structures in *vivo* including preformed K-fibers and spindle midzones in anaphase. Recently we have found that ISB, a subassembly of the CPC composed of $INCENP^{1\text{-}58aa}$, Survivin and Borealin can form liquid demixed coacervates in *vitro* that concentrate free tubulin. Moreover, the CPC coacervates nucleate microtubules at concentrations of free tubulin below the critical concentration and bundle these microtubules into complex structures. We hypothesize that the CPC directly nucleates and bundles microtubules in preformed K-fibers and midzones. We have purified the full *Xenopus* CPC complex from *E. coli* and show that it undergoes phase separation similar to the ISB subassembly. CPC coacervates also induce microtubule nucleation, polymerization, and bundling in *vitro*. This reaction is rapid, and occurs below the critical concentration of tubulin. Activating Aurora B kinase activity by adding ATP generates distinct structures from those generated in the absence of kinase. Prc1 (protein required for cytokinesis 1), the midzone-associated microtubule crosslinker, interacts with microtubules nucleated by the CPC and further organizes them into more complex structures. We suggest the CPC generates nonmembranous organelles at inner centromeres and midzones to nucleate microtubules to generate complex microtubule structures that are essential for faithful chromosome segregation and cytokinesis.

P1105/B231

Critical Roles of a Rhogef-anillin Module in Septin Architectural Remodeling during Cytokinesis.

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Critical roles of a RhoGEF-anillin module in septin architectural remodeling during cytokinesis Xi Chen*, Kangji Wang*, Tatyana Svitkina, and Erfei Bi How septin architecture is remodeled from an hourglass to

a double ring during cytokinesis in fungal and animal cells remains unknown. Here, we show that septins in budding yeast form a striking "zonal architecture" in which paired filaments along the mother-bud axis lay the foundation for the entire transitional hourglass whereas the RhoGEF Bud3, the anillin-like protein Bud4, and circumferential single filaments localize exclusively to the outer zones while the myosin-II filaments occupy the middle zone. Deletion of *BUD3* or its coding sequence for the Bud4-interacting domain, but not the RhoGEF domain, completely abolishes single filament assembly whereas deletion of *BUD4* or its coding sequence for the anillin-homology and PH domains, which also mediate its interaction with Bud3, destabilizes the transitional hourglass, especially at the mother side. This and further analysis indicate that Bud3 stabilizes the single filaments whereas Bud4 fastens the junction between the paired and single filaments. Not surprisingly, deletion of *BUD3* and *BUD4* together abolishes transitional hourglass and double ring assembly. We also demonstrate that the septin double ring is dispensable for cytokinesis unless the actomyosin ring is also compromised. Thus, the two cytokinetic structures, which are pre-patterned by and simultaneously generated from the transitional hourglass, normally act in concert to ensure efficient cytokinesis. This study establishes a mechanism and function for the role of a RhoGEF-anillin module in septin architectural remodeling at the cell division site.

P1106/B232

Aurora B Functions at the Apical Surface After Specialized Cytokinesis during Morphogenesis in *C. Elegans*.

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While cytokinesis has been intensely studied, the way it is executed during development is not well understood, despite a long-standing appreciation that various aspects of cytokinesis vary across cell and tissue types. To address this, we investigated cytokinesis during the invariant *C. elegans* embryo lineage and found several reproducibly altered parameters at different stages. During early divisions, furrow ingression asymmetry and midbody inheritance is consistent, suggesting specific regulation of these events. During morphogenesis, we found several unexpected alterations to cytokinesis including apical midbody migration in polarizing epithelial cells of the gut, pharynx and sensory neurons. Aurora B kinase, which is essential for several aspects of cytokinesis, remains apically-localized in each of these tissues after internalization of midbody ring components. Aurora B inactivation disrupts cytokinesis and causes defects in apical structures, even if inactivated post-mitotically. Therefore, cytokinesis is implemented in a specialized way during epithelial polarization and Aurora B has a new role in the formation of the apical surface.

P1107/B233

Actin-independent, Microtubule-dependent Cytokinesis In*Chlamydomonas*.

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Cytokinesis in unikonts (animals, fungi, slime molds, and their close relatives) involves the "contractile ring", which contains F-actin, myosin II, and other proteins. The force generated by actin-myosin II interaction is widely viewed as the primary driver of cleavage-furrow ingression. However, many types of unikont cells can form furrows even in the absence of normal myosin-II activity. Furthermore, the wide diversity of non-unikont eukaryotes do not possess myosin II, yet most of them divide by furrowing, using mechanisms that are very poorly understood. Thus, we lack understanding of the division mechanism of the last eukaryotic common ancestor and thus of the true role and evolutionary advantage of the contractile ring in the unikonts.To address these questions, we are studying the mechanisms of cytokinesis in *Chlamydomonas*, an alga that has no myosin II yet divides by forming cleavage furrows. We previously showed that although F-actin is enriched in the cleavage furrow, it is dispensable for its ingression. Unexpectedly, division of the large chloroplast was delayed in these cells, suggesting a novel role for F-actin in coordinating the timing and location of cell division and chloroplast division. The furrows formed in the absence of F-actin are still associated with microtubules, and pharmacological dissolution of microtubules completely blocked furrowing, indicating that these cytoskeletal elements, unlike F-actin, are essential for furrow formation. Furthermore, the localization and movements of the plus-end-binding protein EB1 and the minus-end-directed kinesin KCBP suggest the possibility that stable rootlet microtubules located in the furrow region act as non-centrosomal organization centers for nucleation of other, more dynamic microtubules that may be involved in membrane addition to the furrow region, similar to the furrow-associated array in animals and the phragmoplast in plants. The implications of these observations for the evolution of eukaryotic division mechanisms will be discussed.

P1108/B234

The DNA Repair Protein Nopo Has a Mitotic Function That Suppresses Neuronal Stress Response to Prevent Microcephaly.

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Microcephaly is a reduction in brain size caused by a loss of neuronal proliferation and survival. Most genes linked to microcephaly primarily function at either the mitotic spindle or in DNA replication and repair (DRR) pathways, and thus microcephaly is thought to arise via one of two pathways. However, in many cases the precise roles of microcephaly-related genes in brain development have not been studied directly. One such gene is *TRAIP*, which is mutated in a severe form of microcephaly and is well studied for its roles in DRR. Accordingly, *TRAIP* microcephaly is thought to arise via delays in S-phase and accumulation of DNA damage. We characterized *nopo*, the *Drosophila* homolog of *TRAIP*, and found a novel mitotic function, thereby linking the two microcephaly pathways in a single gene. *nopo* mutants have microcephaly-like defects in a pair of brain structures called the mushroom bodies (MBs). MBs are normally made of ~2000 neurons that arise from four neuroblasts (NBs); *nopo* mutant MBs have reduced size and neuron numbers. Contrary to its canonical role in DRR, we did not find evidence for increased DNA damage in *nopo* mutant MB neurons or NBs. Instead, the MB NBs of *nopo* mutants are

often multi-nucleate, suggesting mitotic failure, and are progressively lost during development via caspase-dependent cell death. Consistent with its established DRR functions, Nopo has nuclear localization during interphase; however, it has dynamic localization in mitosis, streaming along mitotic spindles and concentrating at the cytokinetic furrow. Deleting the nuclear localization sequence (NLS) evicts Nopo from the nucleus during interphase, presumably abrogating any DRR functions; nonetheless, expressing Nopo lacking the NLS fully rescues the *nopo* mutant microcephaly-like phenotype. Together, these results reveal a novel mitotic function for *nopo* that is critical for neurogenesis, thus raising questions about the current model of *TRAIP* microcephaly and whether other microcephaly-related genes also play dual roles. Finally, by screening for downstream pathways in *nopo* mutants, we discovered that inhibiting the neuronal stress pathway MAP3K *wnd*/*DLK* suppresses microcephaly-like phenotypes. Thus, the *wnd*/*DLK* pathway is identified as a novel key factor and is a potential therapeutic target for minimizing neuron loss in microcephaly.

P1109/B235

Physical Integration within *Xenopus* Egg Extract Microtubule Asters Suggests Aster Movement Is Driven by Dynein-dependent Surface Forces and Actomyosin Contraction.

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The eukaryotic cytoplasm includes dynamic networks of cytoskeletal polymers and membranous organelles that mutually interpenetrate and mechanically interact via steric and molecular interactions. During telophase, forces on astral microtubules cause centrosomes to move away from the division plane to partition into daughter cells and position the cleavage furrow. In *Xenopus laevis* eggs, centrosomes travel hundreds of microns away from the midplane after anaphase of first mitosis. This occurs before astral microtubules reach the cortex, so forces must be generated within bulk cytoplasm. Two related questions are unanswered: How are asymmetric forces on asters generated, and do asters move relative to other cytoplasmic components, or collectively with them? Using *Xenopus* egg extracts, we co-imaged microtubules, F-actin, endoplasmic reticulum (ER), mitochondria, and a soluble probe in interphase asters as they grew, interacted, and moved. Aster movement was triggered in two ways: away from boundaries where asters interacted, and by dynein attached to the coverslip, which caused periodic motion under some conditions. Within moving asters, multiple cytoplasmic networks tended to move in the same direction at the same velocity, suggesting physical integration. Photorelease of soluble fluorescein showed that even small molecules are advected with the moving aster network. Dynein-mediated transport did cause inwards movement of ER and mitochondria. Inwards velocity was maximal at aster surfaces, suggesting force generation by organelle-bound dynein is mostly restricted to the aster surface. Centrosomes moved predictably away from aster boundaries, leading to regular spacing of the centrosomes. We expected this movement to be dynein-dependent, but inhibition experiments revealed a large role for actomyosin contraction away from regions of low actin density at aster boundaries. Our results inform a multicomponent active gel model of aster movement, in which dynamic, interpenetrating cytoplasmic networks mechanically interact to separate centrosomes and position the egg cleavage plane.

P1110/B236

Excitable Extract Makes Waves.

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Reconstitution of biological systems is an essential tool for uncovering the molecular mechanisms that underlie cellular function. Here we describe the development of an *ex vivo* system that reconstitutes cortical excitability on a supported lipid bilayer. In cells, cortical excitability is a phenomenon characterized by waves of active Rho (Rho-GTP) and actin polymerization that are thought to "prime" cells to initiate actomyosin-dependent events such as cytokinesis and cell migration. Our group has previously characterized cortical excitability in developing *Xenopus laevis* embryos and starfish oocytes[1]. We found that cortical waves are amplified by overexpression of the RhoGEF Ect2 and inhibited by actin filaments (F-actin). These results suggest that cortical excitability exists as an activator-inhibitor system, where both positive (Ect2-dependent) and negative (F-actin-dependent) feedback loops exist simultaneously to induce wave formation and propagation. We have generated an excitable *ex vivo* system using actin-intact *Xenopus* egg extract on a supported lipid bilayer[2]. These components sufficiently reconstitute a robust wave of Rho-GTP followed by a wave of actin polymerization. The phase shift between the waves of Rho-GTP and F-actin is comparable to in *vivo* cortical waves. This single wave, however, is quickly extinguished by the formation of a dense network of bundled actin filaments, which inhibits continued wave propagation. We find that adding a drug that inhibits actin polymerization creates an environment where multiple waves of Rho-GTP can travel, unobstructed, on the bilayer. These propagating waves of active Rho have a regular periodicity and exhibit patterning that closely resembles F-actin waves in *Xenopus* embryos. This work raises new questions about F-actin-mediated negative feedback in cortical excitability; for example, how is active Rho "turned off" in the absence of actin polymerization? Additionally, the excitable extract system provides a powerful environment for investigating the role of RhoGAPs, select lipids (e.g. $PI(4,5)P_2$, $PI(3,4)P_2$), as well as other downstream effectors (Anillin, Myosin II), in mediating negative feedback that regulates cortical wave patterning. 1. Bement *et al*. (2015) Activator-inhibitor coupling between Rho signaling and actin assembly make the cell cortex an excitable medium. *Nat Cell Biol*. 17(11): 1471-1483. 2. Nguyen *et al*. (2014) Spatial organization of cytokinesis signaling reconstituted in a cell-free system. *Science*. 10; 346 (6206):244-7.

P1111/B237

Intramolecular Regulation of an illin during Cytokinesis.

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Cytokinesis describes the separation of a cell into two daughters, which occurs due to the ingression of an actomyosin ring. Cytokinesis must occur with high fidelity to avoid fate changes and aneuploidy. Recently, we uncovered a novel mechanism whereby the ring protein anillin is regulated by importins during cytokinesis. Importins bind to cargo proteins that contain nuclear localization signals (NLS) and mediate their transport into the nucleus. Active Ran is enriched in the nucleus and competes with importins to release the cargo. During mitosis, the Ran gradient persists around chromatin, where it regulates spindle assembly. The dogma is that importins bind to the NLS of spindle regulators to block interactions with partners required for their function. Thus, active complexes assemble the spindle near

chromatin, where active Ran is high. Our data supports a very different function for the regulation of cortical proteins by importin-binding. We found that importins function as a ruler during cytokinesis, where optimal levels facilitate anillin's cortical recruitment. This mechanism permits precise sensing of chromatin position to ensure that the contractile ring is properly positioned. Here, we determined how importin-binding regulates anillin's cortical enrichment. An illin has a RhoA-binding domain (RBD) required for recruitment to the equatorial cortex, and we found that the RBD autoinhibits the adjacent NLS-containing C2 domain. Our model is that binding to active RhoA causes a conformational change in anillin that increases accessibility to the C2 domain, and importin-binding stabilizes this change for cortical recruitment. Our data supports this model. We found that active RhoA facilitates importin-binding, while inactive RhoA or mutating the RBD decreases importin-binding. Through live-imaging and FRAP experiments we found that mutating the NLS alters anillin's cortical properties. An illin's localization and function are abolished when NLS mutations are combined with mutations that weaken the interface between the RBD and C2 domain. Stronger interface mutations similarly fail to localize to the furrow and rescue loss of endogenous anillin. This data shows that the interface is crucial to drive feedback between the C2 domain and the RBD for recruitment to the equatorial cortex. We propose that other cortical proteins could be regulated by importin-binding during mitotic exit, and to reconsider the dogma of how importin-binding could impact protein function.

P1112/B238

Detection of Key Proteins of the Chromosomal Passenger Complex in Different Phases of Mitosis.

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The accurate and timely orchestration of chromosomal, cytoskeletal events regulated by the chromosomal passenger complex (CPC) leads to seamless cell division. CPC is a heterotetrameric complex consisting of an enzymatic component Aurora B along with three regulatory components INCENP (Inner centromere protein), Survivin and Borealin which are responsible for the activity and the localization of the Aurora B kinase. The CPC dynamically localizes to different subcellular locations to regulate key mitotic phases such as correction of kinetochore-microtubule attachment errors, activation of the mitotic spindle assembly checkpoint as well as assembly and maintenance of the outer kinetochore. Loss of CPC function results in lagging chromosomes, segregation errors and cytokinesis failures. This promotes genomic instability, which contributes to oncogenesis by altering the balance of critical growth and death pathways, along with the overall expression of oncogenes and tumor suppressors. Given their importance, it is imperative to have antibodies that specifically recognize these proteins and work in different functional assays. Towards this we have developed &/or validated antibodies against Aurora B, Plk1, INCENP and Survivin in relevant cell models, applications (Western blot and Immunocytochemistry) as well as confirmed for specificity using cell treatment, relative expression (different phases of cell cycle) and knock-down approach. The data demonstrates that during cell division, CPC localizes along the chromosome arms and at centromeres during prophase. It is restricted to the inner centromere region from prometaphase to metaphase. During anaphase, it moves to the central spindle microtubules and finally accumulates in the mid-body region at telophase thus establishing differential subcellular localization of CPC proteins during different phases of mitosis.

P1113/B239

Cytoskeletal Networks Inhibit Contractile Ring Assembly and Ingression.

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The GTPase RhoA is a central regulator of cytokinesis in metazoan cells. Failure to activate this GTPase blocks cleavage furrow formation. Conversely, local RhoA activation induces the formation of an ingressing furrow. RhoA activation can be induced with a high level of spatiotemporal control in any region of the cell cortex with a light-controlled probe that modulates recruitment of RhoGEF to the plasma membrane (Wagner & Glotzer, 2016). Local RhoA activation suffices to induce a furrow in anaphase cells, mitotic cells, and rounded interphase cells. However, light-mediated RhoA activation fails to generate a cytokinetic furrow in adherent interphase cells with strong actin networks present. To determine whether cell adherence or the presence of cytoskeletal networks interferes with the ability of a cell to assemble an ingressing contractile ring in response to optogenetic RhoA activation, we generated adherent interphase HeLa cells lacking robust actin networks. Upon activation, adherent cells with robust cytoskeletal elements accumulated additional actin and myosin in pre-existing networks, but lacked the ability to form a furrow. In contrast, adherent cells lacking stress fibers were able to build contractile rings and ingress. These results indicate that pre-existing actin networks may block the formation of a contractile ring, and that cell rounding during mitosis and cytokinesis may enhance the disassembly of cytoskeletal structures, thereby permitting contractile ring assembly.

P1114/B240

The Kinetic Landscape and Interplay of Protein Networks in Cytokinesis.

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Cytokinesis is a complex process involving force production, membrane trafficking, and extracellular matrix (ECM) remodeling. While individual proteins have been analyzed for their roles in cytokinesis in different model systems, the behaviors and interplays of protein networks involved in distinct aspects of cytokinesis have not been analyzed in any system. To address this question, we attempt to determine the accumulation kinetics of all major cytokinetic proteins in budding yeast using quantitative live cell imaging. We quantified more than twenty GFP-tagged core cytokinetic proteins involved in each of the functional modules such as assembly and constriction of the actomyosin ring (AMR), and formation and degradation of ECM. Strikingly, individual proteins involved in different functional modules showed module-specific patterns, reaching their peaks at the division site in a strict temporal order. These data suggest that cytokinesis is accomplished by distinct and sequentially acting modules. Proteins that are known to function in two different modules displayed a peak between the peaks of the two modules. For example, Iqg1 (yeast IQGAP) is known to function in AMR assembly and ECM formation, and Iqg1 reached its peak between the peaks of Myo1 and Chs2, which play an essential role in AMR assembly and ECM formation, respectively. Thus, the unique accumulation kinetics of Iqg1 likely reflects its function in coupling AMR to ECM formation. To determine the interplays between protein networks, we deleted *CYK3* (*cyk3*Δ), which encodes a putative activator of Chs2, and then monitored its impact on ECM formation (Chs2) and AMR constriction (Myo1). We found that the localization kinetics of Chs2 in *cyk3*Δ was comparable to that of Chs2 in wild-type cells until the peak, but disappeared significantly slowly, which was coupled with delayed AMR constriction. This phenotype was also observed in the *chs2* mutant whose catalytic activity is substantially reduced. This result supports the postulated molecular function of Cyk3 and indicates that a defect in PS formation can feedback to AMR constriction. Taken

together, our study indicates that proteins involved in cytokinesis are organized into distinct functional modules and connectors, each with a unique accumulation profile at the division site. This comprehensive analysis is significant not only for understanding cytokinesis at the system level, but also for predicting functions of those proteins with a defined accumulation pattern at the division site during the cell cycle.

P1115/B241

Understanding Central Spindle Assembly and Maturation in the *C. Elegans* Embryo.

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Cytokinesis in animal cells is driven by constriction of an equatorial actomyosin contractile ring that is dependent on the central spindle, an antiparallel bundled array of microtubules that forms between chromosomes in early anaphase and matures to form the midbody. The central spindle has at least two functions in cytokinesis: the early central spindle controls contractile ring constriction and the final mature midbody serves as a template for abscission, the final step of physical separation of the daughter cells. While the early stages of central spindle formation (microtubule nucleation and bundling) have been well studied, less is known about how the central spindle changes morphologically throughout cytokinesis and forms the mature midbody. In the one-cell *C. elegans* embryo, early central spindle formation is controlled by CLASP^{CLS-2}, which is localized by BUB-1 and CENP-F$^{HCP-1/2\ and}$ nucleates central spindle microtubules just after anaphase onset, and by microtubule crosslinking protein PRC1$^{SPD-1\ and}$ the centralspindlin complex, which bundle central spindle microtubules. Using genetic mutants as a sensitized background, we are currently taking a targeted, candidate-based RNAi approach to identify molecules that transform this early central spindle into the midbody and promote cytokinesis. This work will reveal novel insights into how this dynamic signaling hub forms and supports successful cell division.

P1116/B242

Type 2 Interphase Node Proteins Operate in Tandem to Localize Sid2p to the Contractile Ring for Timely Completion of Cell Division.

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The conserved NDR-family kinase Sid2p is the terminal member of the Septation Initiation Network (SIN) signaling pathway that localizes to the contractile ring during fission yeast cytokinesis to promote ring constriction, septation, and completion of cell division. Previous studies have found that the Type 2 interphase node proteins Blt1p, Gef2p, and Nod1p contribute to localization of Sid2p and its regulatory protein Mob1p at the division site. This in turn promotes constriction of the contractile ring and completion of septation. However, their relative contributions and whether they operate in the same or parallel pathways has been unclear. In this study, we quantify the respective roles of Blt1p, Gef2p, Nod1p, and Klp8p in Sid2p/Mob1p recruitment and characterize the effect of single, double, and triple deletion mutants on contractile ring dynamics and completion of cell division. We find that the Type 2 interphase node proteins work in the same pathway to localize the NDR-family kinase Sid2p and its regulatory partner Mob1p to the division site, thereby promoting timely completion of cell division.

P1117/B243

Coarse-grained Simulations of Cytokinetic Node Protein Mid1 and Path to Dimeric Structure.

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The anchoring of the cytokinetic ring at the cell equator in animals and fungi depends crucially on the anillin scaffold proteins. During fission yeast mitosis, annilin-related Mid1 binds to the plasma membrane and helps establish cytokinetic nodes in a broad band near the cell center. Recent experiments revealed that Mid1 consists of a globular domain with two potential regions for membrane binding and an N-terminal intrinsically disordered region that is regulated by phosphorylation. We used the available structural information in molecular dynamics simulations with an established coarse grained potential to explore the possible modes of Mid1 binding to the membrane and its dimerization/multimerization properties. The coarse-grained model used represents each residue by a single interaction site located at the Cα atom. Mid1 globular domains were treated as rigid bodies fixed in their known crystallographic structure. Disordered regions were treated as flexible and simulations reproduced prior measurements of their radius of gyration. Replica exchange simulations of the ordered regions identified possible binding modes of the Mid1 dimer. This informed a Mid1 membrane-bound model, which was shown to preferentially reinforce the dimer binding mode seen in prior X-ray crystallography data. Fixing binding modes of interest and adding in disordered regions allowed us to investigate how membrane-bound Mid1 may fill space in nodes and compare these results to recent super-resolution node measurements. In the future, this method may help resolve Mid1 structural interactions in nodes.

G1, G1-S, and S Phase Regulation

P1118/B244

An Engineered System for Measuring Multimeric Nuclear Protein Interaction Dynamics in Live Cells Enables a Quantitative Understanding of the Cell-cycle Entry Decision.

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Protein-protein interactions are a critical regulatory point for many cellular processes. Characterizing interactions between proteins has traditionally been performed through biochemical techniques. However, this approach neglects contributions from the cellular environment. Fluorescence recovery after photobleaching (FRAP) can be used to quantify biomolecular interactions in living cells, but measurement of interactions between freely diffusing proteins is not amenable to FRAP. Herein, we present a generalizable method for quantifying interactions between nuclear proteins in live cells. To achieve this, we tethered one interaction partner to an engineered, stabilized nuclear lamin construct and fluorescently labeled other interaction partners, enabling FRAP-based measurement of off-rates. Moreover, we demonstrate that our technique can be used for both chemical and genetic perturbations of protein interactions. We therefore present a powerful method for achieving a quantitative understanding of complex cellular-level interaction networks. We applied this method to systematically measure off-rates between the cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitory proteins (CIPs) that regulate G1-phase cell-cycle progression. We found that both the CDK4/6-cyclin D interaction and CDK2-cyclin E interaction are moderately strong, and interaction strength is greatly increased in the presence of CIPs. Interestingly, we also detected transient binding of CDK2 to cyclin D1. We then investigated how various disease-causing mutations in cyclin D1 and p16 affect CDK4 binding,

correlating interaction strength to cellular phenotype. Finally, we analyzed how inhibition of major growth-signaling pathways can alter G1 regulator protein-protein interactions. Thus, our method enabled a quantitative molecular understanding of how major G1 regulatory proteins dictate cell-cycle entry decisions.

P1119/B245

Mitogen-independent Cell Cycle Progression in B Lymphocytes.

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Clonal expansion determines effectiveness of adaptive immune responses where activation of lymphocytes leads to selection of a tiny proportion of lymphocytes to proliferate robustly and execute the immune program. Proliferation studies in lymphocytes are carried out with persistent mitogenic stimulation, presumably reflecting our bias that mitogenic re-stimulation is necessary to get robust clonal expansion. The classical view, obtained largely from population analyses of fibroblasts, posits that a mitogenic signal is required at the end of each mitosis for cells to progress through the next G1 phase. However, recent analyses of tumor cell lines have shown that a subset of cells within a population retains the ability to undergo G1 progression without additional signaling. For B lymphocytes the germinal centers (GC) proliferation shows good evidence of mitogen-independent proliferative phase in dark zone (DZ). Even, CD8+ T cells have been shown to have similar properties, suggesting that this may be a common feature of cell cycle regulation in lymphocytes. Despite its uniqueness and likely physiological relevance, this form of proliferation has received scant attention. Our study characterizes mitogen-independent proliferation in primary murine B lymphocytes. We demonstrate that, regardless of the initiating stimulus, commitment to DNA replication (S phase) programs B cells to undergo several rounds of cell division in the absence of overt mitogenic signaling and the extent of division is limited by cell death rather than by return to quiescence. High dimensional cytometry identifies mitogen-independent cell cycle progression is driven by unique characteristics of the G1 phase of cells that have divided once, large cell size, low levels of p27 and phosphorylated-Rb. In contrast to studies in cell lines, B cell division under these conditions requires CDK4/6 activity to traverse the second G1 phase. Transcriptional and protein analyses revealed up-regulation of survivin (Birc5) in the G1 phase of B cells past first mitosis and pharmacological inhibition of survivin function blocked G1 progression of cells undergoing mitogen-independent proliferation, but not of naïve B cells stimulated with mitogens. These observations indicate that, in contrast to textbook models of the cell cycle, B cells inherit a partially active G1 phase after cell division that permits them to move quickly to the next S phase in the absence of exogenous G1 progression signals. We propose that these mechanisms may assist in rapid cell division without differentiation that is required for clonal expansion in response to antigen and in B cell proliferation in the GC-DZ.

P1120/B246

Understanding the Effects of Atrazine on Human Keratinocytes.

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Atrazine is the second most used herbicide in the United States and the most common chemical detected in American groundwater. Despite being banned from various European countries, the EPA has deemed concentrations below 3.0 parts per billion (ppb) to be safe for use. Atrazine can enter the body via inhalation, ingestion, or penetration where it is then converted into metabolites in the bloodstream. Many studies have labeled atrazine as a carcinogen due to the reproductive defects it causes in rats, fish, and amphibians at levels as low as 0.1 ppb. In 2009, the herbicide was observed as being a potential cause of birth defects and reproductive complications in humans (Pathak, Dikshit; 2011). Some reports have also shown that atrazine causes mitochondrial dysfunction in human liver cells (HepG2). While it is known that atrazine can have an impact on many organ systems, its effect on the skin remains understudied. The importance of this research is underscored by the fact that the skin is our first line of defense and primary means to combat toxins, ultraviolet radiation, and mechanical insults. The goal of this research is to identify whether exposure to atrazine could promote a cancerous phenotype in human keratinocytes. We hypothesize that higher levels of atrazine may act as a carcinogen, affecting cell cycle, adhesion, and morphology.To identify this effect, keratinocytes were exposed to varying concentrations of atrazine that exceeded the EPA's standards. Immunofluorescence was employed to visualize DNA double-stranded breaks with the marker γH2AX, a version of the H2A protein that composes the histone octamer and gets phosphorylated upon DNA damage. Additional cell cycle markers and DNA damage pathways were investigated in keratinocytes treated with atrazine. Our preliminary data suggests that that keratinocytes treated with increasing atrazine concentrations induces DNA damage, alters cell morphology, and causes changes in cell adhesion.Knowledge gained from this work will provide new insights into a previously understudied area.

P1121/B247

A Heterogeneous Nuclear Ribonucleoprotein (hnRNP) Homolog in Chlamydomonas Functions as a Cell-cycle Repressor in the Retinoblastoma Cell-size Control Pathway.

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Coordination of growth and division in eukaryotic cells is thought to be mediated by cell-size checkpoints, but the mechanisms for size homeostasis are largely unknown. The green alga *Chlamydomonas reinhardtii* divides by a multiple fission cell cycle, where two control points are used to couple cell size to cell cycle progression: the Commitment checkpoint ensures that enough growth has taken place to allow completion of at least one division cycle, and the DNA synthesis/mitosis (S/M) checkpoint ensures mother cells undergo the correct number of divisions to produce uniform-sized daughters. The *tny1-1* mutant was identified in a forward insertional mutagenesis screen and exhibits a recessive small-size phenotype due to defects at both the Commitment and the S/M size checkpoints. *TNY1* encodes a predicted hnRNP A-related RNA binding protein with two N-terminal RNA recognition motifs (RRMs) and a low complexity glycine-rich C-terminus, a structure shared by many other of eukaryotic hnRNPs. Immunofluorescence showed that TNY1 is cytosolic throughout the cell cycle. Immunoblotting revealed that daughter cells are born with a fixed amount of TNY1 whose absolute

abundance remains constant on a per-cell basis during G1 phase, but whose overall cellular concentration decreases as cells grow. *TNY1* mRNA and protein levels peak during cell division and are reset to their highest concentration in newly-formed daughters. Altering the dosage of TNY1 in diploids or by overexpression impacted daughter cell size, indicating a quantitative relationship between TNY1 protein levels and mitotic size control. Epistasis experiments placed TNY1 upstream of the cyclin dependent kinase CDKG1 [1], whose substrate is the MAT3/RB (retinoblastoma tumor suppressor homolog). CDKG1 is produced just before cells divide and is eliminated upon mitotic exit, but in *tny1-1* strains post-mitotic CDKG1 remains detectable, suggesting that TNY1 inhibits CDKG1 production or turnover. North-Western assays showed that TNY1 binds to the unusually long and uridine-rich 3' UTR of *CDKG1* mRNA but not to its CDS or 5' UTR. Taken together, our data suggest a model where TNY1 influences cell-size homeostasis through dosage-dependent repression of the activator CDKG1, possibly mediated through direct binding of TNY1 to the *CDKG1* 3'UTR. Experiments to test this model are ongoing. [1] a new class of cyclin dependent kinase in Chlamydomonas is required for coupling cell size to cell division. (2016). eLife 5, e10767.

P1122/B248

Human Cdc14A and Cdc14B Affect Cell Cycle Progression through Their Impact on Ciliogenesis.

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Budding yeast Cdc14 phosphatase has a central role in cell cycle regulation, most prominently in mitotic exit and cytokinesis. Puzzlingly, a uniform picture on the function of the three human hCDC14 paralogues hCDC14, hCDC14B and hCDC14C in cell cycle control has not emerged to date. An explanation for this deviation may be the functional redundancy of the three phosphatases or the only partial depletion of the hCDC14 phosphatase by siRNA in human cells. To address these possibilities, we tested for the expression of *hCDC14* phosphatases *and* analysed cell cycle progression of cells with single and double deletion of *hCDC14*. Our data suggest that *hCDC14C* is not expressed in human RPE1 and HCT116 cells excluding a function of hCDC14C in both cell lines. Deletion analysis of *hCDC14A*, *hCDC14B* and *hCDC14AB* knockout (KO) RPE1 cells indicates that both phosphatases are not important for global cell cycle regulation. However, *hCDC14A* and *hCDC14AB* KO cells show reduced staining for the proliferation marker Ki-67, reflecting a delay in S phase entry. This delay was caused by the formation of elongated cilia in *hCDC14A* and *hCDC14AB* KO cells. In contrast, *hCDC14B* KO executed S phase faster. An increase in the number of ciliated *hCDC14B* KO cells was identified as the cause for the faster S phase progression. *hCDC14A* and *hCDC14B* KO cells both showed an increase in DNA damage. *hCDC14A*, *hCDC14B* and *hCDC14AB* KO cells did not affect mitotic exit and cytokinesis. Thus, loss of function of hCDC14A and hCDC14B only mildly affects cell cycle progression suggesting that human hCDC14 phosphatases function in other cell processes, such as ciliogenesis.

P1123/B249

Persistent Dna Repair Signaling during Mitosis Facilitates Acentric Dna Segregation.

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Cells must promptly respond to DNA damage to maintain genome stability. DNA breaks occurring in actively cycling cells frequently lead to DNA damage checkpoint-triggered cell cycle arrest prior to the next cell division. However, some DNA breaks do not trigger these checkpoints. More recently, cellular

responses to DNA breaks that persist into mitosis have been appreciated, but their molecular regulation remains to be fully determined. Uncovering regulation of these responses is crucial, as failure of such responses can lead to micronuclei, which are aberrant nuclear structures formed from persistent broken DNA that are attributed to disease phenotypes. We have established *Drosophila melanogaster* rectal papillar cells (hereafter papillar cells) as a highly accessible model to study how broken chromosomes segregate and avoid micronucleus formation during mitosis. We have previously identified that papillar cells lack active interphase checkpoints and therefore frequently enter mitosis with broken chromosomes. Strikingly, these aberrant chromosome segregate properly despite lacking centromeres/kinetochores. In addition to having inactive interphase checkpoints, we previously showed that papillar cells accumulate persistent DNA breaks during S-phases that occur several days before mitosis. This long delay between DNA breakage and mitosis allows us to easily investigate interphase and mitotic DNA breakage responses separately. Our prior work[1] revealed that conserved Fanconi an emia proteins underlie micronucleus prevention in dividing papillar cells. Here, we report that DNA damage induced during S-phase results in the robust recruitment of homologous recombination repair machinery in papillar cells. However, unlike in cells with active interphase checkpoints, which repair double-stranded DNA breaks within 24 after damage, damaged papillar chromosomes retain specific DNA homologous recombination proteins for several days. These repair proteins are required for papillar cell survival following DNA breakage. Further, these same repair proteins are not present immediately after mitosis, suggesting that repair of broken DNA may initiate in the first interphase after mitosis. We propose that DNA damage which escapes cell cycle arrest requires initiated, but not completed, repair via homologous recombination in order to segregate broken chromosomes. We hypothesize that this mechanism is relevant to any cell with inactive cell cycle checkpoints or in cases where DNA damage persists into mitosis. 1. Bretscher and Fox (2016), *Dev Cell* 37(5):444-57.

P1124/B250

Probing the Mesoscale Rheology of the Metaphase Spindle.

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Measuring the viscosity and characterizing the overall structure of the metaphase cytoplasm is critical to understanding spindle mechanics. However, probing this space has been challenging due to a lack of tools that could enter the spindle region with minimal disruption. Here, we study the motion of mesoscale Genetically Encoded Multimeric (GEM) particles during metaphase. Our measurements reveal that GEM diffusivity is ~ 40% higher in the metaphase cytoplasm (0.75 ± 0.13 $\mu m^2/s$) as compared to interphase. To test whether GEM diffusion is driven by the movements of motor proteins during metaphase, we study the anomalous nature of particle trajectories in control cells and after pharmacological perturbations. We further characterize the spatiotemporal variations in cytoplasmic viscosity, including those which are normally associated with materials near the glass transition point. Collectively, these results will classify the physical nature of the metaphase cytoplasm and further our understanding of the forces which must be overcome by the metaphase spindle.

P1125/B251

Size-Dependent Expression of the Cdc13 and Cdc25 Mitotic Activators Redundantly Regulate Cell Size in Fission Yeast.

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The coordination between cell growth and division is a highly regulated process that is intimately linked to the cell cycle. Despite a wealth of knowledge about cell growth and division, little is known about the mechanisms that regulate the cell cycle in response to cell size. Efforts to identify a signaling system that measures cell size and conveys that information to the cell-cycle machinery have been unsuccessful. Instead, we propose that size control is an intrinsic function of the basic cell cycle machinery. Using both population-level and single cell assays we have shown that two key positive regulators of the G2/M transition—Cdc13, the B-type cyclin that forms CDK with the Cdc2 kinase, and Cdc25 [1], the phosphatase that activates CDK—accumulate in a size dependent manner. That is, unlike most proteins which maintain a constant concentration as cells grow, Cdc13 and Cdc25 increase in concentration as cells get bigger. Crucially, we have shown that Cdc13 and Cdc25 accumulation is directly proportional to size (i.e., they act as sizers), as opposed to being proportional to the amount of time cells stay in G2 (i.e., they do not act as timers). Since both Cdc13 and Cdc25 are dose-dependent activators of mitosis, their size-dependent accumulation provides a mechanism by which cells can regulate their size [2]. We have shown that Cdc25 is regulated transcriptionally [1]. We have gone on to show that a 100-bp promoter is sufficient to drive size-specific expression of Cdc25. This promoter contains motifs conserved within the promoters of the three other fission yeast species. We will present evidence that this promoter could regulate size-dependent expression via a nuclear-sites-titration model. Cdc13, on the other hand, is regulated translationally or post translationally. Its mRNA maintains a constant, size-independent concentration, but produces a size-dependent concentration of protein. We have localized the determinants of size-dependent expression to the 5' UTR or the ORF. We will continue to narrow down their location, be they in the transcript or protein. 1) Keifenheim et al., (2017) Size-Dependent Accumulation of the Mitotic Activator Cdc25 Suggests a Mechanism of Size Control in Fission Yeast. Current Biology 27(10):1491-1497<https://www.ncbi.nlm.nih.gov/pubmed/28479325> 2) Rhind, (2018) Cell Size Control Via an Unstable Accumulating Activator and the Phenomenon of Excess Mitotic Delay. Bioessays 40(2):1700184<https://www.ncbi.nlm.nih.gov/pubmed/29283187>

P1126/B252

E2F-dependent Genetic Oscillators Control Endoreplication.

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Polyploidy is an integral part of development and is associated with cellular stress, aging and pathological conditions such as cancer. Across many organisms, the endoreplication cycle, comprised of successive alternations of G and S phases without cell division, is commonly employed to produce polyploid cells. To maintain the endocycle, oscillation of Cyclin E (CycE)/Cdk2 activity is required, where low Cdk2 activity during G phase allows pre-replicative complex (pre-RC) formation and high Cdk2 activity triggers origin firing. Previous studies have identified E2F1 as one of the core molecular machineries that drives the biphasic G-S oscillations. In this study, we provide mechanistic insight on how endoreplication cycles are maintained by E2F proteins. Specifically, we show that the biphasic oscillation of E2F1 and CycE requires the recently described alternate isoform of E2F1, E2F1b, which is

necessary for development of polyploid tissues in Drosophila. The impact of E2F1b deregulation was analyzed using the Fluorescent Ubiquitination-based Cell Cycle Indicator (FUCCI) in actively endoreplicating larval salivary glands. Genetic experiments revealed that E2F1b regulates the circuitry of timely S phase entry and exit during endoreplication by activating a subset of E2F-target genes. E2F1b regulates the Drosophila ortholog of CDK inhibitor Dacapo (Dap) to precisely time S phase entry by controlling the CycE/Cdk2 activity threshold. Dap-mediated inhibition of CycE/Cdk2 allows G phase cells to accumulate CycE and pre-RC components, such as Double Parked/Cdt1, prior to entering S phase. Upon entry to S phase, E2F1b-dependent burst of Proliferating Cell Nuclear antigen (PCNA) expression establishes a negative feedback loop through the PIP box-mediated degradation of E2F1. This mechanism is critical for ensuring proper downregulation of E2F1 target genes and S phase exit. Overall, our study uncovered a network of E2F-dependent genetic oscillators that are critical for the periodic transition between G and S phases during endoreplication.

P1127/B253

Investigating the Role of the SWI/SNF Chromatin Remodeling Complex in the Differentiation of the Invasive Phenotype.

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The success of many metazoan developmental programs relies on the ability of specialized cells to transgress basement membranes (BMs). Cancer progression also relies on cellular invasion. Though the developmental and clinical importance of cell invasion is evident, studying its dynamics in *vivo* has proven to be challenging. Using high-resolution microscopy, as well as genetic and cell biological techniques, we study the process of anchor cell (AC) invasion into the vulval epithelium during *C. elegans* development as a model for cellular invasion. We have demonstrated that the conserved nuclear hormone receptor transcription factor, NHR-67, is required to maintain the AC in G1/G0 cell cycle arrest, a requirement for invasive behavior. Independent of cell cycle arrest, the AC utilizes the histone deacetylase, HDA-1, for the generation of invadopodia, and the expression of pro-invasive genes. These results suggest that invasion is a differentiated cellular behavior requiring cell cycle arrest and epigenetic modification of the genome. To identify additional chromatin modifiers that mediate invasion, we are conducting a tissue-specific RNAi screen. To date, we have identified several new pro-invasive genes which encode subunits of the SWI/SNF chromatin remodeling complex. *The* SWI/SNF complex is pleiotropic and contributes to cell fate specification in the somatic gonad and cell-cycle exit and differentiation of muscle and intestinal precursors in *C. elegans*. Here, we show a conserved role for the SWI/SNF complex in coordinating cell cycle arrest, as loss of *swsn-1* results in a mitotic AC. We have endogenously GFP-tagged core SWI/SNF subunit SWSN-4 in order to confirm AC expression, and taking advantage of GFP-targeting nanobody technology, we are able to confirm a strong loss of invasion phenotype following SWSN-4::GFP depletion in the AC. Furthermore, we are examining the role of the SWI/SNF complex in maintenance of the post-mitotic state and the regulation of pro-invasive gene expression, through potential interactions with NHR-67, HDA-1, other chromatin modifiers and the cell cycle machinery. Using a DNA Helicase B-based CDK2 biosensor our lab has recently adapted to *C. elegans,* we are investigating the cell cycle state of the AC in chromatin modifier depleted states. Together, these results will provide insight into the role of the SWI/SNF complex in orchestrating invasive activity and coordinating exit from the cell cycle.

P1128/B254

Quantitative Proteomics in G_1 Cells Reveals the Insulin Receptor Adaptor IRS2 as an APC/C^{Cdh1} Substrate.

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The anaphase promoting complex/cyclosome (APC/C) is a ubiquitin ligase that controls progression through the eukaryotic cell cycle by targeting key substrates for degradation through the ubiquitin proteasome pathway. During G_1, the APC/C works in concert with its co-activator Cdh1 to recognize and ubiquitinate specific substrates during this phase of the cell cycle. To identify novel pathways and substrates regulated by APC/C^{Cdh1}, we conducted an unbiased proteomic screen in G_1-arrested RPE1 cells acutely treated with small molecule APC/C inhibitors. Combining these results with degron prediction analysis, we uncovered diverse putative APC/C substrates. We validated IRS2, a key adaptor protein involved in signaling downstream of the insulin and IGF1 receptors, as a novel direct APC/C^{Cdh1} target. We demonstrate that genetic deletion of IRS2 reduces the expression of proteins involved in cell division and functionally impairs the spindle assembly checkpoint. Together, these findings reveal a novel connection between the insulin/IGF1 signaling network and the cell cycle regulatory machinery.

P1129/B255

Assessing How Cells Enter and Exit the Endoreplication Cell Cycle.

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Endoreplication is an alternative cell cycle wherein cells go through alternating G and S phases without completing mitosis. While endoreplication is a normal cellular mechanism that can be used to generate natural polyploid cells, it can also be co-opted by cancer cells and lead to genome instability and tumor evolution. We have developed a method to generate induced endoreplicating cells (iECs) by treatment with cell cycle kinase inhibitors to define the mechanisms that allow entry into and exit from endoreplication. Inhibition of Aurora B kinase resulted in a type of endoreplication called endomitosis in which cells enter mitosis but do not complete division. Cells treated with higher concentrations of inhibitor failed mitosis before the metaphase/anaphase transition, leading to large multi-lobed nuclei in the daughter cells. In contrast, cells treated with lower concentrations of inhibitor had a failure in cytokinesis, resulting in binucleate cells. These results suggest that Aurora B inhibition stops cells from progressing through mitosis in a dose-dependent manner. To ask how cells exit the endoreplication cycle, we generated iECs and used flow cytometry to isolate populations of cells with 2C/4C or 8C DNA and then allowed them to resume mitosis after drug washout. During outgrowth, we saw a lag in proliferation before cells began to rapidly divide and form colonies of mitotically dividing cells. After three weeks, the cells had returned to a diploid state but had increased chromosomal instability. To understand how the iECs resume division, we focused our studies on the critical time period during which proliferation begins. Using proliferation markers, we found that iECs in the lag phase were not Ki-67 positive nor did a significant proportion of cells undergo S phase. We also examined nuclear morphology at two-day time intervals during outgrowth. Initially, all of the cells had large multi-lobed nuclei, but over time we observed a small, but consistent increase in the proportion of binucleated cells. This suggested that binucleation may be an intermediate step between multi-lobed nuclei and the normal nuclear phenotype seen after outgrowth, suggesting that cells may exit endoreplication using the reverse mechanism of how they entered. We are currently using time-lapse imaging to examine the timing and morphology of the cells during the re-entry into rapid proliferation. Ultimately our goal is to

identify the key signaling pathways that facilitate the entry into and exit out of endoreplication to understand how cells use alternative cell cycles to contribute to genome instability and tumor formation.

P1130/B256

Survivable Environmental Change Enhances Drug Survival and Promotes Genome Instability.

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Transient environmental stresses induce signaling pathways that alter cell metabolism to promote survival under changing conditions. DNA replication stress also induces survival mechanisms, primarily the DNA replication checkpoint. Our earlier work using temperature-sensitive alleles of replication proteins, and synchrony through nitrogen depletion, suggested that environmental stress alters cell response to replication stress. To test this prediction, we used fission yeast replication checkpoint mutants including *rad3^{ATR}Δ, mrc1^{CLASPIN}Δ*, and *cds1^{CHK1}*Δ. Under typical lab conditions, these mutants have 1% to 10% relative viability after 4h in hydroxyurea (HU). However, pre-conditioning cells with environmental stress before drug exposure significantly increases replication mutant survival after 4h in HU. Temperature shock, in particular, promotes excellent replication stress survival in mutants. Survival is directly correlated to the duration of temperature treatment, and is linked to adaptive environmental stress response and increased ribonucleotide reductase. Upon return to normal growth temperatures, enhanced HU-survival decays over time and with cell divisions. Yet, this improved temperature/HU-survival in replication mutants comes at a price; surviving cells show increased mutation rates and DNA mis-segregation. While environmental stress alters replication checkpoint response even as mutant cells become less drug sensitive. This altered drug sensitivity has implications for chemotherapy, showing the potential for environmental stress to generate a surviving sub-population that is altered by the now-survivable drug exposure.

P1131/B257

Phosphoregulation Provides Functional Specificity to Biomolecular Condensates in the Cell Cycle and Cell Polarity.

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How cells control the position, timing and lifetime of phase-separated compartments in cells is not fully understood. The RNA-binding protein Whi3 regulates (or contributes to) the cell cycle, cell polarity and stress responses in the multinucleate, filamentous fungus *Ashbya gossypii*. When Whi3 is deleted or mutated, the nuclear division cycle in the continuous cytoplasm becomes more synchronous, the cells have difficulty establishing and maintaining polarized growth, and the cells show heightened sensitivity to certain environmental stresses. Whi3 contributes to these disparate cell processes by its ability to form different complexes with distinct spatial localizations and mRNA targets. For example, CLN3 (a G1 cyclin) transcripts are heterogeneously positioned near nuclei in a Whi3-dependent manner. BNI1 (a formin) transcripts are positioned at growing tips and nascent polarity sites in a Whi3-dependent manner. Additionally, the Whi3 protein can undergo a liquid-liquid phase separation in the presence of target mRNAs in vitro. We are using this system where distinct Whi3 complexes coexist in a single cell to address how, when and where specific identities are established for phase-separated compartments. We hypothesized that local post-translational regulation of Whi3 protein confers specificity to establish these distinct complexes and encodes their function. We found by mass spectrometry that certain sites

on Whi3 are phosphorylated specifically in response to perturbations in the cell cycle or environmental stresses. Creating genetic backgrounds in which these individual sites have been mutated to be phosphomimetic or unphosphorylatable allowed us to find separation of function alleles that are functional for cell polarity or nuclear cycling, but not both. Additionally, some complete loss of function alleles were identified that were still capable of forming condensates showing that phosphorylation is not simply serving as a cue to drive condensation or dissolution. This work furthers our understanding how post-translational modification can be used to regulate distinct populations of phase separated complexes with common components, and is an important part of understanding the physiological role and regulation of these compartments in all cells.

P1132/B258

Dynamic Live-cell Visualization and Quantification of Cell Cycle and Pharmacological Response Using a Genetically-Encoded, Ubiquitination-Based Sensor.

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Monitoring the progression or disruption of the cell cycle is a relevant tool for the advancement of cancer and regenerative medicine research. The ability to view and analyze fluctuations in the cell cycle enables researchers to answer key questions around proliferation, toxicity, and mechanism of action of therapeutic compounds. Standard technologies to monitor cell cycle are often limited to end-point analysis. The IncuCyte® Live-Cell an alysis System is uniquely suited to visualize, monitor, and analyze cell cycle over time. Here we demonstrate the utility of the IncuCyte® Cell Cycle Red/Green Lentivirus Reagent, encoding a FUCCI (Fluorescence Ubiquitination Cell Cycle Indicator) multi-gene expression system to enable live-cell analysis. The sensor was transduced into a panel of cell lines, resulting in stable, non-perturbing expression of green or red fluorescent proteins during S/G2/M or G1, respectively. The IncuCyte® Cell-by-Cell an alysis Software Module was used to quantify subpopulations of cells within each stage of the cell cycle across multiple cell divisions. To study cell cycle synchronization, HeLa cells were treated with thymidine (2.5 mM, 24 h), a DNA synthesis inhibitor, to arrest cell growth in S/G2/M, resulting in 75% of cells expressing green fluorescence. Following release of the thymidine block, image-based measurements were acquired to track the synchronized progression of the cell population as it passed through three 17-hour cycles, as demonstrated by changes in fluorescence: S/G2/M (green), M/G1 (colorless), G1 (red), and G1/S (yellow). To demonstrate pharmacological disruption of cell cycle, a fibrosarcoma cell line (HT1080) was treated with cisplatin or 5-fluorouracil (5FU), resulting in concentration-dependent arrest of the cell cycle in S/G2/M or G1, respectively. Mechanism of action was demonstrated by treating a panel of breast cancer cell lines with diverse hormone receptor patterns (T47D, ER+ & HR+; AU565, ER- & HER2+; MDA-MB-231, TN) with highly targeted compounds. Tamoxifen binds the estrogen receptor and induced arrest only in T47D, while CB-839 showed activity only in triple negative MDA-MB-231 cells. In an additional study to couple observations of drug-induced cell cycle arrest with immune cell morphology and function, THP-1 monocytes were exposed to Phorbol 12-myristate 13-acetate (PMA; 100 nM). PMA-induced differentiation resulted in a decrease in cell proliferation and increase in cell area, along with an increase in percentage of cells expressing red fluorescence, indicative of cell cycle arrest in G1. Overall, these data highlight the utility of the IncuCyte Cell Cycle Red/Green Lentivirus Reagent to provide valuable kinetic measurement of cell cycle phase distribution by live-cell analysis.

P1133/B259

Cdc55-PP2A Dephosphorylation of Pds1 Is Required for Maintaining Genome Stability during Cellular Replication Stress.

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Genomic stability is essential for normal cellular development, protecting the cell from potential tumorigenesis or cell death. In the presence of replication stress or DNA damage, checkpoint pathways become activated to halt the cell cycle. Cyclin-dependent kinase (CDK) governs the cell cycle and its activity is highest during S and M phase. Mitotic exit pathways play a key role in CDK inactivation and the phosphatase PP2A(Cdc55) is a main component of these pathways. Cdc55, a protein kinase, is a component of the regulatory B-subunit within the PP2A complex. In *Saccharomyces cerevisiae*, securin (Pds1) is an anaphase inhibitor and plays an essential role in DNA damage and spindle checkpoint pathways. Pds1 becomes stabilized in response to DNA damage via phosphorylation by Chk1 (checkpoint kinase 1), a conserved serine/threonine kinase. We found that cells deficient in Cdc55 were highly sensitive when treated with the DNA synthesis inhibitor hydroxyurea (HU) and exhibited delayed recovery following HU release. We further showed that the elimination of Pds1-Chk1 phosphorylation sites in a cdc55 mutant greatly reduces HU sensitivity while the deletion of Chk1 along with Cdc55 resulted in a partial suppression of HU sensitivity. Furthermore, we were able to show that Pds1 levels remained stabilized in a Cdc55 mutant following HU treatment. Our results indicate that PP2A(Cdc55) may dephosphosphorylate Pds1 to allow the recovery from cell cycle arrest induced by a DNA synthesis block.

Chromosome Organization 2

P1134/B260

Binding of Methylated H3 by TopoisomeraseIIa Is Required for Resolving Tangled Genomic DNA during Mitosis for Faithful Sister Chromatid Disjunction.

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TypeII DNA Topoisomerase (TopoII) is an essential enzyme that decatenates tangled genomic DNA for sister chromatid disjunction through its unique Strand Passage Reaction (SPR) activity. Loss of the TopoII SPR activity results in the formation of DNA bridges and/or Polo-like Kinase Interacting Checkpoint Helicase (PICH) coated ultra-fine bridges (UFBs) - indicators of flawed sister chromatids disjunction. Vertebrates carry two isoforms- TopoIIα and TopoIIβ, known to share a conserved catalytic core. However, TopoIIα is indispensable during mitosis and its function cannot be compensated by TopoIIβ. TopoIIα's exclusive function in mitosis is attributed to it by its distinct C-terminal domain (CTD) that is dispensable for SPR. In this study, we demonstrated that specific binding of TopoIIα to methylated histones governs the key difference between TopoIIα and TopoIIβ function in mitosis. TopoIIα-CTD contains the Chromatin Tether (ChT) domain at the end of its CTD that specifically interacts with methylated Histone H3. Using the Auxin Inducible Degron (AID) system in combination with the Tet-inducible expression of recombinant TopoIIα, we discovered that the ChT domain of TopoIIα plays critical role in resolving PICH - coated UFBs during mitosis. Together with the in vitro chromatin binding assay using TopoII-CTD fragments, we identified critical residues that governs TopoIIα specific function

in binding to methylated H3 and resolving UFBs. Taken together, we propose that TopoIIα-CTD recognizes specific methylated H3 - associated chromatin to facilitate the resolution of tangled genomic DNA during mitosis for proper sister chromatid disjunction.

P1135/B261

The Role of Phosphorylation in the Elasticity of the Tethers That Connect Separating Chromosomes throughout an aphase.

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Elastic tethers stretch between the telomeres of all separating partner chromosomes during anaphase in animal cells. As anaphase progresses the tethers become less elastic. Our experiments look at the possible role of phosphorylation in regulating tether elasticity. Calyculin a (CalA), an inhibitor of phosphatases PP1 and PP2A, added to crane-fly spermatocytes during anaphase was reported to cause backwards movement of some chromosomes after they reached the poles. We assume the backwards movements were due to tethers. Since tethers become inelastic as anaphase progresses, we tested whether CalA would cause backwards chromosome movement when added after tethers were already inelastic. We inhibited dephosphorylation of tethers of different lengths by adding CalA to living crane-fly spermatocytes at various points in anaphase. When 50 nM CalA was added to cells with short tethers, in early anaphase, almost all chromosomes moved backwards after nearing the poles. When 50 nM CalA was added to cells when the tethers were longer, in mid anaphase, fewer chromosomes moved backward, and when the tethers were yet longer, in late anaphase, none moved backward. This suggests that tether elasticity is lost when tethers become dephosphorylated by either PP1 or PP2A. To distinguish between tether dephosphorylation being caused by PP1 versus PP2A we treated cells with okadaic acid. While 50 nM CalA blocks PP1 and PP2A equally well, the same concentration of okadaic acid blocks solely PP2A. A concentration ~100 times higher is needed in order to block PP1. Only the higher concentration of okadaic acid added to the cell when the tethers are short caused chromosomes to move backward. Thus, it appears that tether elasticity is moderated by dephosphorylation caused by PP1.

P1136/B262

Casein Kinase I Protein Hrr25 Regulates Sister Chromatid Cohesion during Mitosis in *Saccharomyces Cerevisiae*.

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Eukaryotic cell division requires the precise execution of an orderly sequence of cellular events to ensure the faithful separation of sister chromatids into daughter cells. One of the most important aspects of chromosome segregation depends on the cohesin complex that keeps the sister chromatids together until their separation at anaphase. The mitotic cohesin complex consists of four subunits, namely, Scc1, Scc3, Smc1, and Smc3. The establishment and maintenance of the cohesin complex in mitosis requires a number of proteins during and post DNA replication, including acetyltransferase Eco1, Ctf18/Dcc1/Ctf8 alternative replication factor C (Ctf18-RFC) clamp loader complex, cohesion maintenance protein Pds5, and chromatin-associated protein Ctf4. In budding yeast, Hrr25 is a protein kinase that has been implicated in many cellular processes including vesicular trafficking, ribosome biogenesis, autophagy, transcriptional regulation, meiosis, and DNA damage response. While the function of Hrr25 during meiosis has been well established, much less is known about its role in mitotic

cell division. Here we show that an *hrr25* mutation is synthetic lethal with mutations in *CTF4*, *CTF18*, *DCC1*, *CTF8*, *SCC1*, *SMC1*, *PDS5*, and *ECO1*. We further analyzed mutations in about 40 genes for synthetic interactions with an *hrr25* mutation, including those encoding proteins involved in DNA replication, microtubule function, spindle checkpoint, DNA damage checkpoint and DNA repair, and the gene encoding the histone 2A variant, *HTZ1*. We found that an *hrr25* mutation is synthetic lethal with mutations in genes encoding Cik1 and Kar3 that are required for the chromosome bipolar attachment to mediate sister chromatid separation. Additionally, we found an *hrr25* mutation leads to synthetic-slow growth defects with mutations in *HTZ1* and *CIN8*, encoding a kinesin motor protein, but not with mutations in genes encoding other kinesin-related proteins and dynein. Finally, we directly observed defects in sister chromatid cohesion in *hrr25* mutant cells. Together, our data suggest that Hrr25 plays an important role in chromosome segregation during mitosis.

P1137/B263

Numa Is Required for the Formation of a Single Nucleus After Mitosis.

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Eukaryotic cells pack their genome into a single nucleus at the end of mitosis, and failure to do so leads to DNA damage and loss of genomic integrity. However, how mammalian cells form a single and robust nucleus remains poorly understood. NuMA is a long coiled-coil protein essential for spindle structural stability and chromosome segregation. Impaired NuMA function results in nuclear fragmentation, and whether this is due to impaired mitotic function or to a direct role in nuclear formation remains unclear. Here, we show that NuMA plays a *bona fide* role in the formation of a single nucleus after mitosis, independent of its spindle role. We further demonstrate that NuMA is essential for forming a round, smooth nucleus, suggesting a structural role. We find that its coiled-coil is essential to the formation of a single nucleus, and to its stable incorporation in its nuclear structure. Finally, we show that the cell actively regulates NuMA's chromosome binding: NuMA's C-terminus binds chromosomes at interphase but its long coiled-coil prevents it from binding chromosomes at mitosis, keeping its action on distinct cellular structures compartmentalized. Together, our findings indicate that NuMA plays key, long-range structural roles throughout the cell cycle, keeping both the mitotic spindle and the nucleus intact.

P1138/B264

Extra Crossovers Negatively Impact Meiotic Chromosome Segregation in *C. Elegans* Oocytes.

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During meiosis, there is one round of DNA replication followed by two sequential divisions, enabling diploid organisms to cut their chromosome number in half to generate haploid gametes. This process depends on reciprocal recombination, or crossing over, between homologous chromosomes, which holds homologs together in chiasmata so that they can segregate to opposite spindle poles during the first meiotic division. In most organisms, the number of crossovers between homologs is limited by a mechanism called crossover interference, but why this interference process is so well conserved is not known. To understand the importance of crossover interference, we sought to determine whether extra crossovers affect the fidelity of the meiotic divisions in *C. elegans.* This organism exhibits strong

interference, with only one crossover per chromosome pair, resulting in a cruciform bivalent organized around a single chiasma. The bivalents then condense, and a complex of conserved proteins forms an essential ring-shaped structure around the interface between the homologs (the ring complex/"RC"), which is required for proper chromosome congression and segregation. Utilizing a *C. elegans* strain containing an end-on fusion of three chromosomes, we found that a large percentage of the fusion chromosomes had additional crossovers that led to extra chiasmata. At diakinesis, these fusion chromosomes exhibit abnormal chromosome structural patterning and multi-lobed bivalent structures in comparison to the normal cruciform shape. Subsequently, assembled RCs frequently appeared fragmented on these misshapen bivalents, and bivalents with altered RC shape were more likely to be misaligned on the spindle, suggesting that the structure of the RC affects its function. In an aphase I, the fusion chromosomes frequently exhibited segregation defects and a high prevalence of chromatin bridges; although sometimes these bridges were able to resolve, in other cases the bridges persisted, creating a "tether" between the incompletely-extruded polar body and the Meiosis II spindle. Depletion of LEM-3, a late-acting nuclease, increased the prevalence of these bridges, and persistent bridges were preferentially extruded during the second meiotic division, suggesting that there are both universal and oocyte-specific mechanisms for resolving anaphase bridging. These findings demonstrate that excess crossovers can severely impact chromosome patterning and segregation, highlighting the importance of limiting the number of recombination events between homologous chromosomes in meiosis.

P1139/B265

CDK-1 Controls Meiotic Chromosome Dynamics through Phosphorylation of the Synaptonemal Complex.

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Dramatic reorganization of chromosomes is essential for their proper segregation during meiosis. Prior to the first meiotic division, chromosomes pair, synapse, and recombine with their homologs to form the bivalent structure necessary for homolog segregation. The Polo-like kinases play widely conserved roles in orchestrating these processes. In *C. elegans*, PLK-2 is initially localized to pairing centers to promote pairing and synapsis. PLK-2 then relocates to the synaptonemal complex (SC) on the "short arm" relative to the crossover site by binding an SC component SYP-1 and drives a chromosome remodeling, ultimately defining where cohesion will be released during meiosis I. However, the mechanisms governing the dynamic behavior of PLK-2 have remained elusive. Here, we demonstrate that the cyclin-dependent kinase CDK-1 directs the recruitment of PLK-2 to the SC through phosphorylation of SYP-1. CDK-1 phosphorylates a conserved motif within SYP-1 along the entire length of the SC upon meiotic entry. However, pairing centers prevent the recruitment of PLK-2 to the SC until crossovers are designated. We find that the nucleoplasmic protein HAL-2 preferentially targets PLK-2 to pairing centers and that mutating the CDK-1 phosphorylation site on SYP-1 partially rescues PLK-2 targeting to pairing centers in *hal-2* mutants. Finally, we provide evidence that crossover formation is signaled in *cis* to enrich for SYP-1 phosphorylation and that this requires the kinase activity of PLK-2. Taken together, our work establishes a novel role for CDK-1 in orchestrating meiotic chromosome dynamics and provides a mechanism for dynamic PLK-2 targeting during meiosis.

P1140/B266

An Evolution-Guided Approach to Identifying Interaction Interfaces in the Synaptonemal Complex.

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The Synaptonemal Complex (SC) is a proteinaceous structure that is essential for sexual reproduction. The SC ensures that offspring inherit the correct number of chromosomes by aligning parental chromosome pairs during meiotic prophase and regulating exchanges between them. Previous studies have shown that the SC consists of a few (<6) proteins which assemble along the length of paired meiotic chromosomes with a ladder-like ultrastructure. However, despite the essential role of the SC in meiosis and the conservation of its ultrastructure, SC subunits are thought to be dynamic and undergo constant rearrangements. This has led to the hypothesis that SC subunits may assemble by forming multivalent interactions, whereby each protein-protein interaction is weak or labile. As such, traditional techniques have had limited success in describing SC protein-protein interactions. We took an evolution-guided approach to determine protein-protein interaction interfaces in the SC in *Caenorhabditis elegans*. We identified a conserved five amino acid domain in the N-terminus of SYP-1 (one of the four SC proteins in *C. elegans*), and used CRISPR/Cas9 to randomly mutate each of the five residues in the endogenous *syp-1* locus. One mutant, *syp-1*^{mut1}, exhibited hallmarks of meiotic perturbation. Microscopy studies showed that *syp-1*^{mut1} fails to fully synapse a subset of chromosomes in most meiotic nuclei and loads abnormally onto unpaired homologs. Additionally, *syp-1*^{mut1} is temperature sensitive. Mutant animals are nearly sterile at 25ºC but produce near wildtype levels of progeny at 15ºC (wildtype animals are fertile at 15 and 25ºC). We reasoned that *syp-1*^{mut1} was defective in interacting with other SC proteins and that this interaction could be restored via compensatory (suppressor) mutations. To identify suppressors of *syp-1*^{mut1}, we performed saturating mutagenesis of *syp-1*^{mut1} with N-ethyl-N-nitrosourea and selected for suppressor animals that were fertile at 25ºC. We identified 23 suppressor strains and subjected each to mapping and whole genome sequencing. This revealed two revertants to wildtype *syp-1*, and 21 suppressors representing 11 additional point mutations in SC proteins. Importantly, both the original mutation and the majority of the suppressor mutations alter charged residues. Our results point to a multi-valent interaction interface between several *C. elegans* SC proteins, governed by charge-charge interactions that is essential for proper homolog synapsis.

P1141/B267

Cryo-ET an alysis of the Yeast Synaptonemal Complex in *Situ*.

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The synaptonemal complex (SC) is the large proteinaceous scaffold that assembles between homologous chromosomes by the end of meiotic prophase. While its functions are numerous -- and mysterious, in yeast the SC is required for the phenomenon known as crossover interference. Knowledge of the native structure of this complex is needed to evaluate how the SC carries out its functions. Traditional electron microscopy and super-resolution light microscopy have revealed that in many organisms, the SC has a ladder-like structure: two rail-like lateral elements are bridged by a set of rung-like transverse filaments. The filaments are connected along their centers by a central element. To determine the 3-D architecture of the SC and nuclear macromolecular complexes in situ, we studied frozen-hydrated meiotic yeast cell cryosections by Volta electron cryotomography and subtomogram analysis. We find the predominant SC motif is a duster-like arrangement of densely packed triple-helical filaments, both of which are also abundant in the polycomplexes of pachytene-arrested cells. There was no evidence for a ladder-like organization. Partial dissolution by 1,6-hexanediol treatment suggests that these triple-helical filaments belong to the central region of the SCs and are most likely the abundant Zip1 transverse element protein. Subtomogram averaging revealed that the SC's triple helix is up to 12-

nm thick and has a rise of 5 nm and a pitch of 130 nm. Polymers thinner than the triple helix, such as single or double strands, were not detected; this observation is consistent with the strong self-oligomerization properties of SC proteins. The dense packing of SC subunits supports the notion that the SC's mechanical properties help coordinate the rapid end-to-end communication across synapsed chromosomes. Finally, our study provides a 3-D framework for understanding the other macromolecule machines of meiosis in *situ*.

P1142/B268

Identification of Novel Synaptonemal Complex Components in *C. Elegans*.

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Proper chromosome segregation during meiosis requires that chromosomes pair and undergo crossover recombination with their homologs. In most eukaryotes, crossover formation depends on the assembly of the synaptonemal complex (SC), a zipper-like protein scaffold that forms between paired homologs. The SC is a tripartite protein structure consisting of two parallel stretches of lateral elements that are held together by a central element. The SC central region in *C. elegans* is known to be comprised of four coiled-coil domain proteins, SYP-1, SYP-2, SYP-3 and SYP-4. The four SYP proteins were identified more than a decade ago, and it has been widely believed that they represent the entire set of SC components. Here we report the identification of two new SC components, SYP-5 and SYP-6. SYP-5 and SYP-6 localize along the SC between the two chromosome axes, and their recruitment depends on chromosome axes and other SC components. Using single-molecule localization microscopy, we demonstrate that SYP-5 and SYP-6 transverse the width of the SC in a head-to-head manner with the two N-termini at the center and the C-termini facing the chromosome axes. Interestingly, SYP-5 and SYP-6 are paralogous to each other and play redundant roles in synapsis, providing an explanation for why these proteins have not yet been identified by previous genetic screens. We further find that SYP-5 and SYP-6 contain stretches of low complexity amino acid sequences in their C-termini and that truncating these regions leads to aberrant synapsis and disruption of crossover control. Together, our findings establish SYP-5 and SYP-6 as *bona fide* components of the SC and will provide crucial insights into the mechanisms of SC assembly and its role in crossover regulation.

P1143/B269

A Time-Resolved Network of Meiotic Chromosome Associated Proteins.

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Meiosis, a hallmark of sexual reproduction, reduces the ploidy of a cell by undergoing two chromosome segregations after a single round of chromosome replication. In order to accomplish this diploid-to-haploid transition, homologous chromosomes identify one another and physically associate through DNA recombination, before segregating from one another in the first meiotic division. Meiotic recombination initiates with programmed double strand breaks (DSB) on each chromosome, some of which are eventually resolved as inter-homolog crossovers. Many protein complexes are involved in meiotic recombination, and their spatial and temporal interplay is critical to the proper segregation of chromosomes in Meiosis I. The proteinaceous chromosome axis and synaptonemal complex organize

chromosomes and control their recombination, while other recombination complexes shuttle a subset of double-strand breaks to a crossover fate. Previous studies have globally interrogated the timing of meiotic protein expression and indeed, many individual meiotic complexes have been studied in detail. However, a comprehensive view of meiotic proteins' chromosome localization and interactions has not been performed. Here, we present a protocol to assess the localization of proteins to chromosomes throughout a synchronous *S. cerevisae* meiosis using chromatin-enriched fractions coupled with high resolution tandem mass spectroscopy. We demonstrate the ability of our assay to identify proteins from critical meiotic complexes such as the chromosome axis, synaptonemal complex, ZMM family, recombination complexes, and proteins responsible for resolving key crossover intermediates. By disrupting individual proteins from these stratified groups, we elucidate how meiotic complexes work together to manipulate meiotic chromosomes and promote homolog pairing and segregation during Meiosis I. With this tool we have created a temporal atlas for meiotic proteins, and defined how protein complexes assemble and disassemble from chromosomes to coordinate meiotic recombination.

P1144/B270

A Mutation in NCAPD3, a Condensin II Complex Subunit, Links Mosaic Reversion and Microcephaly to Chromosome Hypercondensation.

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Compaction of chromosomes is essential for accurate segregation of the genome during mitosis. In vertebrates, two condensin complexes ensure timely chromosome condensation, sister chromatid disentanglement, and maintenance of mitotic chromosome structure. Here, we report a heterozygous missense mutation in *NCAPD3*, encoding a subunit of the condensin II complex, in a patient with microcephaly. Microarray and whole genome sequencing of patient saliva and blood revealed there is somatic loss of the disease causing mutation suggesting that revertant cells are under strong positive selection during development. Consistent with this, expression of the mutant protein NCAPD3^{R1411Q} in cultured cells and mice lead to growth restriction. At the molecular level, the mutation acts as a gain of function and mimics loss of phosphorylation at a critical CDK1 phospho-site during mitosis. Previously biallelic loss of function mutations in the condensin complex linked microcephaly to chromosome entanglements. In contrast, NCAPD3^{R1411Q} patient fibroblasts and mice do not display chromosome segregation errors but instead present with chromosome hypercondensation during mitosis. These findings establish chromosome hypercondensation as an additional disease mechanism for microcephaly and suggest that progressive somatic changes can arise as an adaptation to a growth restricting condition.

P1145/B271

A Physical Model of Pairing Chromosomes.

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During Meiosis, each pair of homologous chromosomes must move through the nucleus in order to find each other and become spatially aligned along their entire length, a process known as homologous chromosome pairing. While pairing is taking place, the chromosomes are packaged as long linear polymers constrained to move within the confining nuclear volume. From a polymer physics perspective

this presents a difficult problem: How does the cell manage to align each pair of chromosomes in this dense polymer system, while avoiding interlocks? Our aim is to use a course grained molecular dynamics model to understand homologous chromosome pairing as a physical process. We are particularly interested in questions related to entanglement and interlock resolution during pairing. Our initial simulation data suggests that the length and density of eukaryotic chromosomes places them in the reptation regime of polymer physics (where diffusion is slow), but that fast reptational motion can be driven by the progressive zippering of pairing loci.

P1146/B272

Investigating Distinct Roles of Histone H3 and Histone Variant H3.3 in the *Drosophila* Germline.

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Imbalance between self-renewal versus differentiation of stem cells can result in cancer, tumorigenesis, infertility, or tissue degeneration. Epigenetic mechanisms play a critical role in defining stem cell identity and the process of differentiation, where the epigenome undergoes dramatic remodeling. Therefore, understanding the basis of the mechanisms that regulate epigenomes at a cellular level is integral to better our understanding of how cells acquire distinct cell fates. Our lab studies the epigenetic mechanisms that specify cell fate decisions during asymmetric cell division (ACD) of germline stem cells (GSC). Adult stem cells have the unique capability of self-renewal and ability to differentiate. Our lab utilizes the *Drosophila melanogaster* male germline as a model system to investigate the distinct epigenetic mechanisms that regulate stem cell maintenance and differentiation processes. We use an irreversible DNA recombination to switch expression of histones from “old” to “new”. Intriguingly, histone H3 (H3) exhibits asymmetric inheritance during ACD of GSCs. In contrast, the histone H3.3 variant (H3.3), showed symmetric inheritance. How H3 and H3.3 uniquely define cell fate decisions during ACD is a long-standing question. H3 and H3.3 are 97% identical at the amino acid sequence level. Of the four distinct amino acids in the primary sequence of H3 and H3.3, the role of three amino acids in the main body of histones (HFD) has been well defined. However, little is known about their distinct amino acid located at the N-terminal tail. I utilize established genetics and cell biology methods to systematically study the differences between H3 and H3.3 at their N-termini and examine the role they have in defining stem cell fate. At residue 31 in the N-terminal tail of H3 and H3.3, we generated point mutations to create hybrid proteins of H3 and H3.3(H3A31S encoding H3.3 N-term_H3 HFD and H3.3S31A encoding H3 N-term_H3.3 HFD). My preliminary data suggests that inheritance patterns are altered in GSCs expressing these hybrid proteins. We also observed unique cellular defects in these testes. Collectively, this work will allow us to gain insight into how histone variants regulate complex ACD processes in a well-established metazoan model organism.

P1147/B273

A Protein Complex Essential for *C. Elegans* Meiosis Is Comprised of Distinct Structural Domains.

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During meiosis, a single round of DNA replication is followed by two rounds of division in order to give rise to haploid gametes. Female gametes (oocytes) of most species undergo meiosis in the absence of centrosomes. In *C. elegans* oocyte meiosis, microtubules form lateral associations with the chromosomes instead of end-on attachments to facilitate chromosome movement. Additionally, a ring-shaped protein complex wraps around the central region of the bivalent in meiosis I (MI) and the sister

chromatid interface in meiosis II (MII). The ring complex is comprised of over fifteen known protein components including the Aurora B homolog AIR-2, the kinase BUB-1, the plus-end directed kinesin KLP-19, and the post-translational modification SUMO and its associated enzymes. Ring proteins are required for chromosome congression as well as the loss of sister chromatid cohesion that enables segregation of chromosomes to opposite spindle poles. Despite the clear necessity for the ring complex in *C. elegans* meiosis, how the ring performs its roles in chromosome congression and segregation is poorly understood. Specifically, the relationship between the ring's structural properties and essential functions remains to be explored. Although the ring complex is typically depicted as having a hollow circular shape, our work demonstrates that its structure is more complex. Instead of solely wrapping around the outer surface of the bivalent, super-resolution microscopy revealed that ring components weave through the mid-bivalent region in distinct loop-shaped domains. Notably, these substructures, or "sub-loops," are only present on MI bivalents and not MII chromosomes, suggesting that they associate with the short arm axis that corresponds to the homologous chromosome interface. Many ring components that are found on the outer ring also localize to the sub-loops, including AIR-2, BUB-1, KLP-19, and SUMO. Interestingly, sub-loops appear to undergo structural remodeling as meiosis progresses; while most sub-loop components are offloaded from the sub-loops during metaphase, AIR-2 persists on these structures until anaphase onset. Therefore, we hypothesize that the population of AIR-2 that is in the sub-loops could play a functional role in facilitating homologous chromosome segregation, for example in promoting resolution of the crossover. Ongoing investigation of the sub-loop domains of the ring complex will give us further insight into the relationship between the structure and function of this essential protein complex.

P1148/B274

Failures in Fertility: Elucidating the Function of Gsp-3/4 in *C. Elegans* Meiosis.

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DNA partitioning and segregation during meiosis and mitosis is imperative for cellular proliferation, genetic diversity, and embryonic development. Without such processes, nondisjunction and DNA fragmentation are certain to occur—producing germ cells that are often unable to support life. Meiosis and mitosis are extremely vital to all living organisms and the two systems rely on an extensive network of protein kinases and phosphatases that concertedly activate and deactivate proteins to coordinate these complex pathways. The absence of just one phosphatase belonging to the protein phosphatase 1 (PP1) family has shown to disrupt the products of meiosis and promote DNA fragmentation in mammalian sperm. Other PP1 orthologues have been identified in numerous organisms that, when depleted in germ cells, also disrupt meiosis. It is clear that PP1 phosphatases are necessary for successful meiosis but the mechanism governing this success remains unclear. In this proposal, I aim to elucidate the transient role of two 98% identical sperm-specific PP1 phosphatases, GSP-3 and GSP-4 (further referred to as GSP-3/4) in the context of *Caenorhabditis elegans* meiosis. I plan to accomplish this via co-immunoprecipitation experiments to identify any possible subunits or substrates that interact with each phosphatase as 'key players' in the PP1 phosphatase mechanism. Additionally, I plan to use an auxin-inducible protein degradation system to quantify and compare global phosphorylation levels in both wild-type and GSP-3/4 depleted strains of *C. elegans*. Taken together, these data will both identify novel proteins that associate with GSP-3/4 and quantify the enzymatic activity of each phosphatase to develop a mechanism that describes how GSP-3/4, and other PP1 phosphatases, encourage proper chromosome segregation in meiosis.

Oncogenes and Tumor Suppressors 2

P1149/B276

Sos1 and Sos2 Are Therapeutic Targets That Play Unique Roles in Mutant Egfr-driven Lung Cancer Cells.

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Lung adenocarcinoma is the leading cause of cancer death. Activating *EGFR* mutations are found in ~15% of lung adenocarcinomas and are the major cause of lung cancer in never smokers. In patients harboring either an *EGFR* L858R point mutation or an exon 19 deletion (85% of cases), the first-generation EGFR-TKIs erlotinib and gefitinib enhance progression-free and overall survival, however, disease progression invariably occurs with secondary *EGFR (T790M)* 'gatekeeper' mutations and 'oncogenic shift' to other RTKs accounting for a majority of cases (60% and 15-30% of cases, respectively). For resistant tumors harboring *EGFR (T790M)* mutations, the 3rd generation EGFR-TKI osimertinib further prolongs survival, however resistance to osimertinib invariably emerges. Here again tertiary EGFR mutations, oncogenic shift to alternative RTKs, and mutations of downstream RTK pathway signaling intermediates have been identified as potentially driving inhibitor resistance and tumorigenic growth. Since a majority of EGFR-TKI resistance mechanisms continue to rely on RTK/RAS signaling to drive oncogenesis, we investigated whether the RasGEFs SOS1 and SOS2 represente tractable therapeutic targets for both primary and EGFR-TKI resistant tumors. We found that *SOS1* deletion reduced oncogenic transformation in NSCLC cells that were sensitive or resistant gefitinib and blunted 'oncogenic shift' to other RTKs. Furthermore, combining EGFR inhibition with the novel SOS1 inhibitor BAY-293 showed marked synergy in inhibiting transforming growth of NSCLC cancer spheroids in 3D culture, suggesting that SOS1 inhibition is a potent primary therapeutic target in EGFR-mutated NSCLC cells. In contrast, while *SOS2* deletion alone did not alter EGFR-driven transformation, combining *SOS2* deletion with low dose EGFR-TKI treatment synergized to inhibit anchorage-independent growth. Finally, in *situ* resistance trials in a 96-well plate format showed that deletion of either *SOS1* or *SOS2* delayed the development of osimertinib resistance and reduced the overall incidence of osimertinib resistance. Overall, these data indicate that the RasGEFs SOS1 and SOS2 are important mediators of mutant EGFR signaling, and therapeutic targeting of SOS in combination with EGFR-TKI therapy may have clinical benefit for patients with EGFR-mutated lung cancer.

P1150/B277

Cross-cancer Study with a Special Emphasis on Stomach Cancer Reveals Atad2 as a Diagnostic and Prognostic Biomarker.

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ATAD2, an AAA+ ATPase, has recently been found to be overexpressed in many cancers and correlated with the malignant status of the disease. Therefore, *ATAD2* could be considered as a potential cancer biomarker. By systematic bioinformatics analyses with relevant experimental studies, here we evaluate for the first time the prospect of *ATAD2* as a diagnostic and prognostic biomarker for cancers. Our cross-cancer analyses reveal that *ATAD2* is overexpressed in many cancers (Oncomine and GEPIA (TCGA) databases) and is related to poor survival of the cancer patients (Kaplan-Meier Plotter and SurvExpress

databases). Most interestingly, *ATAD2* appears as the cancer driver *for many cancers* and the potential mutation sites of *ATAD2* (cBioPortal and COSMIC databases) are predicted. All these studies across the cancer types specify ATAD2 as an interesting target particularly for stomach cancer. Therefore, we further focus our study on stomach cancer and indeed find that *ATAD2* is overexpressed in all stages and grades of stomach adenocarcinoma (Oncomine and GEPIA (TCGA) analyses as well as expression analyses with stomach cancer cell lines). Our immunofluorescence microscopy study with adenocarcinoma as well as metastatic stomach tissue samples also shows that *ATAD2* is upregulated in stomach carcinoma; corroborating our finding from cell culture experiments and in *silico* studies. Further survival analysis indicates that high expression of *ATAD2* drastically affects the survival of the stomach cancer patients (Kaplan-Meier analysis). Therefore, we next used cox proportional hazard regression model and receiver operating curve analysis. Our analyses clearly illustrate that high expression of *ATAD2* could be a predictable factor for prognosis of stomach cancer with perfect accuracy. *ATAD2* appears as cancer driver for stomach cancer (iPAC) *and* a total of 40 mutational sites (including the hot spot mutations at ATPase domain) of *ATAD2* have been identified; signifying that many of these mutations could be responsible for stomach oncogenesis. We also show that *ATAD2* directly interact with many transcription factors which could potentially help in ATAD2 mediated cellular proliferation. The top canonical pathways of ATAD2 are identified (ingenuity pathway analysis) indicating the prominent role of ATAD2 in cancer cell proliferation. Therefore, the analysis reported here establishes *ATAD2* as a promising prognostic marker for multiple cancers, and a very promising biomarker and therapeutic target for stomach cancer.

P1151/B278

P53 Amyloid Formation as an Ignition Key for Cellular Transformation: Is Cancer a Prion Disease?

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p53 gene is mutated in more than 50% of human cancers, leading to loss of its tumor suppressive function.p53 mutation results in its misfolding and/or aggregation leading to its sequestration in cytoplasmic/nuclear inclusions and compromising its native function. Mutational landscape of p53 has been systematically characterized in a large cohort of samples but the link between p53 conformational status and its contribution to cancer is largely unexplored. Our group has shown that native, wild type p53 undergoes amyloid formation and these aggregates possess prion-like properties, thereby spreading the p53 aggregation to neighboring cells. Here, we propose that wild type p53 protein can aggregate upon specific stimulus, resulting in loss of p53 function driving the cells towards cancer progression. Using an in *cellulo* model, p53 fibril seeds were used to induce p53 amyloid formation. p53 fibril treated cells demonstrated increased survival and resistance to apoptosis. Treated cells displayed tumorigenic potential evident by enhanced migration and increased colony formation propensity. Cells with p53 aggregates showed an over-expression of Epithelial-mesenchymal transition markers and formed tumor-like spheroids in 3D cell culture. Injection of cells with p53 aggregates induced tumor formation in xenograft mice model whereas no tumors were detected in mice injected with untreated cells. Furthermore, by using immunohistochemistry, these tumors tested positive for p53 amyloid aggregates. Global gene expression and proteomic profiling revealed p53 amyloid formation in cells significantly influences apoptosis, cell cycle and proliferation, chaperone levels, senescence along with major signaling pathways like MAPK and Wnt pathway. Gene ontology analysis further showed up-regulation

of pro-oncogenic pathways and a loss of tumor suppressive pathways thus contributing towards tumorigenic potential in cells. The present study proposes that native, wild type p53 amyloid formation could act as an ignition key for cancer initiation and the prion-like nature of these amyloid species classifies cancer as a prion-mediated disease.

P1152/B279

P21$^{Waf1/cip1}$ Reduces P53 Protein Stability by Facilitating the Action of Wip1 on P53.

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The functions of the p53 tumor suppressor/transcription factor are regulated by cellular components that control the stability of protein p53. ATM, which is activated in response to DNA damage, directly phosphorylates serine-15 (S15) of p53, which stabilizes p53 by preventing its binding to the E3 ubiquitin ligase Mdm2. In contrast, the phosphatase Wip1 dephosphorylates p53-S15, facilitating Mdm2-induced p53 ubiquitination and subsequent proteasomal degradation. As p21$^{WAF1/CIP1}$ binds directly to p53, we investigated whether p21 regulates p53 stability. We found that p21 reduces stability and cellular concentration of protein p53. Such functions of p21 required Mdm2, Wip1, and proteasomal activity. Furthermore, we revealed that Mdm2 requires p21 to induce ubiquitination and proteasomal degradation of p53. Moreover, p21 facilitates dephosphorylation of p53-S15 by Wip1. Thus, p21 may facilitate p53 degradation by facilitating the actions of Wip1 and Mdm2 on p53. Therefore, p21 appears to be a new regulator of p53 stability, which may provide novel insights into the mechanisms underlying tumor progression and DNA damage response.

P1153/B280

Suppression of Human Transposons by P53.

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p53 gene mutation is the most common genetic alteration in human cancer. As a transcription factor, p53 regulates important cellular stress response pathways including apoptosis, cell cycle arrest, and senescence. However, compelling studies from several groups have established that these canonical effectors are not adequate to prevent oncogenesis. Recent findings from our lab uncovered an evolutionarily ancient role for p53 in the containment of mobile genetic elements in the germ line of flies and fish. Retrotransposon activity may contribute to destabilized genomes and inflammatory responses that typify human cancers. Therefore, we proposed to determine whether retroelement repressions are conserved p53 function in humans. First, we created p53 knockout in different human cell lines using Crispr-cas9. Importantly, the LINE1 retroelement encoded L1ORF1-p protein was highly expressed in theses p53 knockout lines when compared to their p53 wild type parental lines. Next, we used histone deacetylase inhibitor treatment (HDACi) to artificially induce expression of LINE1 elements in both p53 wt parental and p53 knockout cell lines. Upon withdrawal of HDACi, the p53 knockout cells failed to re-establish repression of the LINE1 elements. Together our data indicates that p53 is required to for both maintenance and de novo silencing of retrotransposons in human somatic cells. These p53 functions may constitute important, and previously unappreciated tumor suppressive mechanisms.

P1154/B281

Identifying the Mechanism and Impact of Hnrnp K Regulation by Keratin 19.

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Breast cancer is the second deadliest type of cancer among women in the United States. One of the biomarkers that physicians use to predict breast cancer patients' overall survival is Keratin 19 (K19). Keratins are critical for maintaining the structural integrity of epithelial tissues but are also involved in other non-mechanical functions including cell growth, proliferation, and apoptosis. In cancer, higher expression levels of K19 correlate with poor survival of breast cancer patients. However, the exact role of K19 in tumorigenesis is not understood. Our data shows there is an interaction between K19 and RNA-binding protein hnRNP K, which can impact all steps of the gene expression. HnRNP K has been found to be overexpressed in many cancers, and its cytoplasmic accumulation has been correlated with tumor cell growth and metastasis. However, the molecular mechanism of how hnRNP K cytoplasmic localization is regulated in K19 dependent manner needs to be studies. We found that cell proliferation and cytoplasmic localization of hnRNP K in MDA-MB-231 breast cancer cells are K19-dependent. To study the effect of K19 on hnRNP K targets on breast cancer cells, we mapped hnRNP K target RNAs in both parental and K19 KO cells using Photoactivatable-Ribonucleoside-Enhanced Crosslinking and Immunoprecipitation (PAR-CLIP). This cutting-edge biochemical technique helps to identify RNA-binding protein target sequences on a transcriptome-wide scale at nucleotide resolution. Using PAR-CLIP to identify hnRNP K targets and RNA-sequencing of parental and K19 knockout (KO) cells, we found hnRNP K targets are downregulated in K19 KO cells, in particular when bound in the 3'UTR, suggesting a cytoplasmic role for hnRNP K in addition to its canonical nuclear role. Then, we used DAVID, a gene ontology tool, to identify functions from a list of top hnRNP K targets deregulated in K19 KO cells. DAVID analysis showed that the endoplasmic reticulum (ER), cell-cell adherent junctions, and the cell cycle are pathways that are most affected among genes that are dysregulated in K19 KO cells. For these pathways, we hypothesize that hnRNP K could impact levels of mRNA targets through K19 by retaining a part of hnRNP K in the cytoplasm, where it is able to bind and regulate a set of targets that are involved in ER, cell-cell adherent junctions, and cell cycle which have been known deregulated in many cancers. In the absence of K19, these targets are then destabilized and the change in gene expression profiles therefore results in the defect in cells proliferation. By characterizing how K19-hnRNP K interaction impacts the transcriptome of breast cancer cells, we hope to ultimately define novel pathways that can be potential therapeutic targets to combat cancer

P1155/B282

Triple Negative Breast Cancer Cell Exposure to Epigallocatechin-3-gallate Increases Expression Levels of Tumor Suppressor Mir-125a-5p.

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Triple negative breast cancer cell exposure to epigallocatechin-3-gallate increases expression levels of tumor suppressor *miR-125a-5p* Authors: Fatma Abdelaal, Toni-Ann Bravo, Kassie Campbell, Lissette Delgado-Cruzata PhD Polyphenols are chemical compounds found in many plant based foods. They offer many health benefits including protection against type 2 diabetes, heart disease, and some types of cancer. They have been shown to target cancer stem cells and to inhibit metastasis and invasion. Studies have proposed that polyphenols act through several mechanisms including regulating the expression of microRNAs (miRNAs), which are small non-coding RNAs that regulate gene expression by sequence

specific binding of mRNA. One important miRNA in breast cancer is *miR-125a-5p*. Overexpression of *miR-125a-5p* showed significant inhibition of breast cancer cell migration and invasion. Research shows that it may inhibit cell proliferation and encourage cell apoptosis by negatively regulating expression of *BAP1*. In clinical settings, lower levels of *miR-125a-5p* have been shown to be an independent predictor of breast cancer suggesting it has an important role in this disease. It is not known whether polyphenols, such as epigallocatechin-3-gallate (EGCG), can modify levels of *miR-125a-5p* in breast cancer cell lines. Here, we investigate the effect of increasing concentrations of EGCG on the growth of the triple negative breast cancer cell line MDA-MB-468 and whether levels of *miR-125a-5p* change after exposure to this chemical. We treated MDA-MB-468 cells with varying concentrations of EGCG for 24 hours, and carried out the MTT proliferation assay. We extracted total RNA, and used miScript reverse transcription and primer assays with quantitative PCR (qPCR) analysis to determine *miR-125a-5p* levels. We found that proliferation significantly decreased in cells exposed to 0.5 mM EGCG in comparison to 1 µ M (44.0 ± 13.0% vs. 85.3 ± 1.9%, respectively (p =0.023)). *miR-125a-5p* levels increased after treatment with 50 µ M EGCG ($miR\text{-}125a\text{-}5p_{50\mu M}$ = 337.1 ± 57.7% (p =0.025) vs. untreated). This work highlights the potential role of polyphenols such as EGCG as an inhibitor of cancer cell growth possibly by modifying the expression of miRNAs such as *miR-125a-5p.*

P1156/B283

Role of RBPMS Splice Variants in Cisplatin Resistance Ovarian Cancer Cells.

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RBPMS is a RNA binding protein with multiple splicing encoded by the RBPMS gene located in chromosome eight in humans. RBPMS is capable to bind and process RNA molecules. RBPMS also interact with proteins in the nucleus and it can blocks the formation of c-Jun-c-Fos and/or c-Jun-Smad3 complexes in order to regulate gene expression. Three major RBPMS isoforms has been described RBPMS-A (21.8 kDa), RBPMS-B (22.4 kDa) and RBPMS-C (24.2 kDa). Reports have shown that each RBPMS isoform interact with different proteins and RNAs. Results of our laboratory found that RBPMS is decreased in cisplatin sensitive compared with cisplatin resistant cells. Small-interference RNA (siRNA)-based RBPMS silencing decreased the sensitivity of ovarian cancer cells to cisplatin treatment. However, the specific RBPMS isoform responsible of this biological effects have not been elucidated. We overexpressed separately the isoform a and C of RBPMS in the cisplatin resistant ovarian cancer cells, A2780CP20 cells. We performed cell proliferation, cell viability, cell invasion, and in vivo tumor growth in subcutaneous mouse models. Significant reduction in the number of colonies were observed in both isoform a and isoform C of RBPMS compared with control clones. However, clones of isoform a but not the isoform C were more sensitive to cisplatin treatment. Invasion assay showed that the expression of RBPMS a and C decrease the invasiveness capacity of the cells when compared with controls. Furthermore, in vivo tumor growth showed a significant reduction in tumor growth between RBPMS a and C when compared with the controls. These results suggest that both, isoform a and C regulate transcripts involved in cell proliferation and invasion capacity of ovarian cancer cells. However, only the isoform a contributed to the cisplatin sensitivity of ovarian cancer cells. Ongoing work are identifying the RNA and proteins associated with each RBPMS isoform.

P1157/B284

Pathological Trans-lesion Synthesis (TLS): a Mutagenic Driver and Molecular Vulnerability in Cancer.

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There are fundamental gaps in our understanding of how neoplastic cells tolerate intrinsic DNA replication stress and DNA damage while simultaneously accumulating the mutations that fuel cancer progression. Trans-Lesion Synthesis (TLS) is a specialized mode of DNA replication that employs damage-tolerant and error-prone DNA polymerases. Therefore, TLS provides a potential explanation for how neoplastic cells endure diverse genotoxic stresses while mutating their genomes during multi-step tumorigenesis. We have tested the hypothesis that TLS facilitates carcinogenesis by conferring DNA damage tolerance and mutability. We show that: (1) the TLS pathway is pathologically activated by an aberrantly-expressed germ cell protein (Melanoma antigen A4, or MAGE-A4) in many cancer cells. (2) TLS maintains ongoing DNA synthesis and sustains viability in cells experiencing oncogene-induced DNA replication stress. (3) the TLS pathway promotes tumorigenesis and shapes cancer genomes in *vivo*. Taken together these results show that TLS endows neoplastic cells with two important tumorigenic phenotypes namely DNA damage tolerance and mutability. The DNA damage tolerance and mutability acquired during carcinogenesis also allows cancer cells to resist therapy-induced genotoxicity. The identification of pathological TLS as a cancer-specific DNA damage tolerance pathway provides opportunities for new treatments that selectively sensitize tumors to genotoxic therapies yet are innocuous to normal cells.

P1158/B285

3D Culture Conditions Reveal Therapeutic Signaling Vulnerabilities in RAS-mutant Cancer Cells.

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Signaling from receptor tyrosine kinases (RTKs) to the small G protein RAS through the RAS guanine nucleotide exchange factors (GEFs) SOS1 and SOS2 is critical for normal cellular proliferation. Activating mutations in this pathway can drive tumorigenesis, and RAS family genes (H-, N-, and KRAS) are the most frequently mutated human oncogenes, accounting for ~30% of cancers. These cancers are disproportionately responsible for cancer-related deaths in the US, and therapeutic options to target these cancers are limited, indicating that identifying novel therapeutic vulnerabilities is key to developing successful treatments. Using RAS-mutant cancer cell lines, we demonstrate that three-dimensional (3D) culture methods reveal signaling dependencies in cancer cells that are masked by growth in two-dimensional (2D) culture.Using cancer cell lines expressing mutants of the three RAS isoforms, we found that RAS family members have a hierarchical requirement for SOS2 to drive anchorage-independent (3D) transformation, but not anchorage-dependent (2D) proliferation, with KRAS>NRAS>HRAS. Consistent with the idea that upstream activators of wild-type RAS, including RasGEFs, represent potential novel therapeutic targets in KRAS-mutant tumor cells, wild-type RAS isoforms cooperate with KRAS to promote downstream signaling and transformation in these cancers. We found that SOS2 was necessary for receptor tyrosine kinase (RTK)-dependent, WT RAS-mediated, PI3K activation in RAS-mutant cells regardless of mutant isoform. The differential dependence of cells expressing mutated RAS to SOS2 deletion was due to a hierarchical dependence on PI3K signaling to resist anoikis in anchorage-independent (3D) growth conditions. These results demonstrate the importance of choosing the appropriate culture system to identify and test novel targets in RAS-mutant

tumors, including using anchorage-independent 3D growth screens to supplement current 2D screening efforts.

P1159/B286

Excitability of the Ras-pi3k Network Controls the Dynamic Morphology and Transformation in Epithelial Cells.

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The Ras-PI3K signaling network is frequently activated in cancer. Studies in Dictyostelium have delineated the role of excitability of this network in cell migration and some parallels have been observed in human cells. Here we show that the activities of the Ras-PI3K signaling network propagate as coordinated waves, biased by epidermal growth factor, which drive actin-based drive protrusions in MDA-MB-231 and MCF10A cells. The signal transduction events display other excitable features including mutual annihilation of oppositely directed waves, full responses to suprathreshold stimuli, and refractoriness. Acute depletion of PI(4,5)P2 or PI(3,4)P2 activated the network by lowering the threshold/setpoint. Activated Ras acts through PIP3 and mTORC2 to drive cytoskeletal activity while ERK provides negative feedback. Importantly, expression of oncogenic H-Ras and K-Ras leads to dramatic increases in coupled signal transduction-cytoskeletal wave activity. These observations suggest that the transformed state can be viewed as a lowering of the threshold of the Ras-PI3K network.

P1160/B287

Functional Link of Mir-200a and Elk3 to Regulate Metastatic Nature of Breast Cancer Cells.

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ELK3 belongs to the ETS family transcription factor and it has been reported to act as an oncogene to regulate epithelial-mesenchymal transition during metastasis of various cancers including prostate, liver and aggressive triple negative breast cancer cells. Considering that ELK3 is also implicated in the lymphangiogenesis during embryonic development, it is expected that there is a strict window to maintain cellular context dependent proper concentration of intracellular ELK3. Indeed, ELK3 protein level is regulated by multiple steps including transcriptional and post-transcriptional mechanisms.Based on our recent report that ELK3 is highly expressed in aggressive basal type compared to luminal type of breast cancer cells, we questioned what is the underlying mechanism that determines the difference of ELK3 level between basal and luminal breast cancer subtypes. Our analysis showed that the amount of ELK3 in breast cancer cell types is controlled by stepwise regulatory mechanism. Firstly, *ELK3* transcription is controlled by promoter activity and *ELK3* promoter in MCF7 is highly methylated to suppress *ELK3* expression. Then, the expressed *ELK3* transcripts are under second control mechanism by miRNAs targeting to 3'UTR of transcribed *ELK3*. Our result from deletion analysis and mutation analysis demonstrated that miR200a is a primary miRNA to determine the ELK3 level in different breast cancer cell type. We demonstrated that the expression of miR200a and ELK3 is functionally linked to regulate cell migration and invasion of breast cancer cells. These findings provide a previously unknown mechanistic insights into the regulation of ELK3 level in various cancer cell types and it is expected to expand our understanding of ELK3 activity in terms of cancer cell characteristics.

P1161/B288

Downregulation of SCAMP3 Suppresses Inflammatory Breast Cancer Progression through EGFR Signaling.

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Inflammatory Breast Cancer (IBC) is a rare and aggressive form of breast cancer. It accounts for ~2.5% of all breast cancers in the U.S. IBC is characterized by rapid progression and the ability to invade the lymphatic system by the formation of emboli and subsequent metastasis development. Epidermal Growth Factor Receptor (EGFR) is overexpressed in IBC and contributes to IBC progression and reduced overall survival. Studies associate the endocytosis related protein, Secretory Carrier Membrane Protein 3 (SCAMP3), with the regulation of EGFR recycling and degradation. Recently, we published that SCAMP3 is overexpressed in IBC cells, tumors and emboli. Others have identified SCAMP3 as an indicator of poor prognosis in hepatocellular carcinoma. Therefore, we hypothesize that SCAMP3 promotes trafficking of EGFR, induces EGFR signaling and IBC progression. To evaluate whether SCAMP3 has a direct interaction with EGFR, we treated IBC cells with the EGFR ligand, EGF (100ng/mL) at different time points, then we evaluated the localization of both proteins by immunofluorescence. Results showed that SCAMP3 co-localizes with EGFR at the cytoplasm at early stimulation times and at the perinuclear area after 30 mins. To evaluate if SCAMP3 has a role in IBC progression, we pre-treated IBC cells (WT) and SCAMP3 knockout IBC cells with EGF to assess cell proliferation, tumor emboli formation, and the EGFR signaling cascade. Our results showed that KO of SCAMP3 decreased proliferation and area of formed emboli. The expression of ERK1/2 was also reduced in SCAMP3 KO cells. Taken together, SCAMP3 is involved in the internalization of EGFR, cell proliferation, and tumor emboli formation. We conclude that SCAMP3 has an essential role in EGFR trafficking and IBC progression.

P1162/B289

Functional Role of the Cell-adhesion Molecule Metadherin in Inflammatory Breast Cancer Progression.

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Inflammatory breast cancer (IBC) is one of the most challenging and lethal forms of primary breast cancer with a 5-year survival rate of 43%. The lethality of IBC originates from its nature of invading the vascular and lymphatic systems, absence of a typical tumor mass, and the generation of tumor emboli. Challenges in this disease include the accurate and early diagnosis and the development of effective targeted therapies. Previous studies investigated potential biomarkers involved in tumor spheroid integrity by characterizing IBC's cell surface proteome. Plasma membrane proteins were identified via "stable isotope labeling with amino acids in cell culture" (SILAC) with subsequent mass spectrometry (MS)-based quantitative proteomics analysis using the IBC cell line SUM-149 vs. non- cancerous mammary epithelial cells, MCF10A. SILAC results showed overexpression of Metadherin (MTDH) in SUM-149 cells (>2.0 fold change). Subsequently, we validated the overexpression of MTDH in SUM-149 cells, IBC tissues and emboli. MTDH is a cell adhesion molecule overexpressed in many cancer types, including breast cancer. MTDH promotes cancer progression by modulating various signaling pathways

including NF-kB, which is related to cell survival, proliferation, invasion and metastasis. Therefore, the aim of this study is to assess the functional role of MTDH in IBC progression. To elucidate the mechanism of action of MTDH in colony formation and tumor spheroid we silenced MTDH (CRISPR or shRNA) the SUM-149 and SUM-190 IBC cells. We assessed the expression of MTDH by immunoblotting and we observed a reduction of protein abundance. The number of colonies formed in SUM-149 MTDH silenced cells was ~40% lower when compared to non-silenced cells. Tumor spheroids of SUM-149 and SUM-190 MTDH silenced cells were smaller than non-silenced cells. Aditionally, MTDH silencing results in a decrease in the phosphorylation of the p65 subunit of NF-kB while no changes are observed in total protein abundance in SUM-149 cells. We can conclude from our preliminary results that MTDH serves as a potential IBC target for IBC progression. This work was supported by NIH NIGMS #GM111171 (MMM), SGRP 2017-00143 (MMM and GOS), NIMHD #MD007583 (MMM), GM103475 (UPR MMM), Title-V-PPOHA #P031M105050, Title-V-Cooperative #P031S130068 from the U.S. Dept. Of Education and Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health #P20GM103475 (GOS).

P1163/B290

Radiation Signaling through P53 Modulates Biological Outcome in Normal Adult Human Endothelial Cells Following Radiation.

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Our laboratory determined that the primary response of normal adult endothelial cells to ionizing radiation (IR) is accelerated senescence. We demonstrated that adult human and bovine pulmonary artery endothelial express senescence-associated β-galactosidase (SA- βgal), upregulated cell cycle checkpoint proteins, and increased levels of anti-apoptotic proteins in response to 5-50 Gy (2.4 Gy/min) IR. The signaling differences that result in accelerated senescence vs apoptosis in response to IR are not completely understood. We hypothesized that p53, a protein activated by redox stress and DNA damage, directs early signal transduction following IR, and that specific pathways downstream of p53 determine the biological outcome. We examined the early signaling events of human lung microvascular endothelial cells (HLMVECs) after high dose IR. Similar to other adult endothelial cells, HLMVECs responded to 10 Gy (1.15 Gy/min) IR mainly by undergoing premature senescence as indicated by SA-βgal expression. We also observed upregulation of cell cycle checkpoint protein p21/waf1 by western blotting, a further indication of senescence. Using qPCR, we examined the expression of genes known to be increased in transformed cells in response to IR. We found a significant increase in insulin-like growth factor receptor 1, an anti-apoptotic tyrosine kinase, and a decrease in PIK3CA and PIK3CB, catalytic subunits of an immune response kinase. We also identified transcriptionally independent upregulation of p21, ATM, phospho-ATM, and phosphor-MDM protein levels increased at 4 h post-IR. We investigated p53 binding partners and target genes using co-immunoprecipitation and western blotting. P53 binding of HIPK2, a conserved serine/threonine kinase, increased significantly within 10 minutes of IR. In order to better understand these results and the difference between transformed and HLMEC p53 signaling and gene regulation, we examined total p53 binding partners using co-immunoprecipitation

with mass spectrometry, and RNA-seq. Our data suggest that signaling in HLMVECs differs significantly from cancer cell signaling in response to radiation, which impacts the biological outcome.

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Tumor Microenvironment

P1164/B291

Transfer of Lipid from Cancer-associated Fibroblasts Enhances Stemness Properties of Cancer Cells Under Glucose Deficient Condition through Stearoyl-coa Desaturase.

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Cancer-associated fibroblast (CAF) plays a crucial role in the tumor progression by interacting with cancer cells. Under tumor microenvironment like glucose deficient condition, lipid metabolism becomes more active for the cancer cell biology. This study investigated the effect of CAF-derived lipids on the cancer cell phenotypes. For xenograft tumor, NCI-H460 (H460) cells were subcutaneously injected into immune deficient mice. Fibroblasts were isolated from the resultant tumor mass and the underarm skin tissue by magnetic-activated cell sorting with anti-FAP and anti-CD90/Thy1 antibodies. Lipid components were measured by a commercial EZ-free fatty acid assay kit and Bodipy staining. Western blot, RT-PCR, immunofluorescence and flow cytometry were performed to assess the expression level of lipid metabolism, cancer and intracellular structure related markers. Mitochondrial function was evaluated using JC-1 assay, Rhod-2AM staining and ATP production assay. Stearoyl-coa desaturase (SCD) expression was manipulated by lentiviral vector and CRISPR/Cas9-based gene editing. Under glucose deficient condition, H460 cells co-cultured with CAFs showed enhanced cell viability and cisplatin resistance compared to those with normal fibroblasts (NFs). The amount of lipid components was higher in CAFs than in NFs. The CAF-derived lipid component was transferred to H460 cells and metabolized by increasing SCD expression under glucose deficient condition. For the lipid supplementation study, oleic acid (OA) was treated to H460 cells. Enhancement of cancer stemness by the supplement of OA to glucose starved cells was significantly mitigated by the treatment with SCD inhibitor. Moreover, cells with upregulated SCD showed significant enhancement of cancer stemness compared to their corresponding controls, but it was remarkably suppressed in SCD-knockout (SCD-KO) cells. Markers of mitochondrial function and intracellular filamentous structure were regulated by SCD expression. Intriguingly, increased F-actin expression in SCD upregulated cells induced the nuclear translocation of YAP, but not in Mock and SCD-KO cells. In xenograft assay, the size of the resultant tumor mass was significantly increased by injection of SCD upregulated cells compared to that by Mock cells. However, SCD-KO cells showed limited tumor development. In conclusion, these results suggested that targeting lipid metabolism in glucose starved cancer cells may be a potential way of anti-cancer therapy, through the suppression of cancer stemness.

P1165/B292

Investigation of Nutrient Sharing between Stromal Cells and Cancer Cells in a Mouse Model of Pancreatic Cancer.

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Pancreatic ductal adenocarcinoma (PDAC) is characterized by tumors that contain cancer cells embedded in a vast excess of non-cancerous, stromal cells. Many of the stromal cells in these tumors are a type of fibroblast called pancreatic stellate cells (PSCs). Using a three-dimensional, in *vitro*, mouse organoid model of PDAC, we have developed conditions such that the PDAC cancer cells require the presence of PSCs to proliferate. PSCs secrete large amounts of collagen, which forms the basis of the extensive extracellular matrix in these tumors. It has been shown that the PDAC cells are able to take up collagen produced by the PSCs as a source of amino acids to support proliferation, however dissecting the metabolic relationships between cancer cells and stromal cells in mixed culture is challenging. As a way to study this, we have engineered PSCs to overexpress the RBKS gene, which encodes ribokinase, an enzyme that catalyzes phosphorylation of ribose to ribose-5-phosphate, thus trapping ribose in cells, where it can serve as a precursor for synthesis of nucleotides. Mammalian genomes possess the RBKS gene, but it is not typically expressed at a level that supports detectable ribokinase activity; thus, cells do not incorporate isotope-lableled ribose as assessed by gas chromatography/mass spectrometry analysis of polar metabolites. We are investigating the ability of cells overexpressing RBKS to incorporate labeled ribose as a way to track label from one cell type to another and dissect how nutrients are shared between PDAC cancer cells growing in co-culture with PSCs.

P1166/B293

The Nutrient Microenvironment of Tissues and Tumors Affects the Metabolism of Resident Cells.

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The metabolism of mammalian cells is influenced both by cell-intrinsic factors and cell-extrinsic factors. There has been substantial effort towards understanding how cell-intrinsic factors, such as disease causing genetic mutations, alter cellular metabolism. However, much less is known about how physiological microenvironmental conditions alter cell metabolism. Nutrient availability is a cell-extrinsic factor that substantially influences cellular metabolism, yet we have relatively little information regarding nutrient availability in different healthy or diseased tissues. To address this, we developed a quantitative metabolomics approach to measure nutrient levels in the interstitial fluid (IF) of tissues and tumors, providing insight into the metabolic substrates available to cells in their local microenvironment. Profiling IF nutrient levels in different tissues and tumors has provided insight into nutrients that are depleted or accumulate in different anatomical sites in the body. To determine how local nutrient availability alters cellular metabolism, we have developed a cell culture medium based on observed IF nutrient levels in murine pancreatic cancers. Pancreatic cancer cells, as well tumor stromal cells, can be grown in IF-based medium in order to study how cells utilize metabolism to support homeostasis and function when constrained by microenvironmental nutrient levels. In initial experiments, we found that freshly isolated murine pancreatic cancer cells grow robustly in IF-based media, but cancer cells initially cultured in standard media lose the ability to grow in IF-based media, suggesting that the adaptations required by cancer cells to cope with tumor nutrient levels are rapidly

lost by pancreatic cancer cells in standard culture. We are using a multi-omics approach to identify what metabolic adaptations pancreatic cancer cells use to support their growth in their nutrient environment. Collectively, these experiments provide insight into how nutrient availability acts as a cell-extrinsic factor to alter pancreatic cancer cell metabolism, and provide an experimental approach for determining how nutrient availability impacts cellular metabolism in healthy and diseased mammaliantissues.

P1167/B294

Breast Cancer Autophagy and Chemoresistance Are Regulated by Substratum Stiffness.

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Breast cancer relapse can develop over the course of years due to dormant cancer cells that at one time disseminated to secondary sites. These dormant cells are often resistant to conventional chemotherapy. Although recurrence is the main cause of death from cancer, the microenvironmental factors that determine the duration of dormancy are largely unknown. Considering that breast cancer relapse is often detected in tissues that are softer than the normal mammary gland and the primary breast tumor, such as the bone marrow, the brain and the lung, we explored how the stiffness of the tissue at secondary sites regulates tumor dormancy and the response of breast cancer cells to chemotherapy. We found that substratum stiffness regulates proliferation and apoptosis of MCF7 human breast cancer cells. Soft substrata with compliances reminiscent of those at secondary sites harbor tamoxifen- and paclitaxel-resistant breast cancer cells as determined by EdU incorporation, TUNEL staining, and immunoblotting for p21, p27, and cyclin D1. To determine why cells survive in soft microenvironments, we investigated the effects of tissue stiffness on autophagy, a process that has been implicated in the survival of dormant cancer cells. Immunofluorescence staining for LC3B, immunoblotting for p62, and autophagosome turnover assays demonstrate that autophagy is increased on soft substrata. Additionally, we found that autophagy contributes to tamoxifen resistance and that blocking autophagy sensitizes MCF7 cells to tamoxifen, especially on soft substrata. We also found that increasing the expression of integrin-linked kinase, which transmits mechanical signals received by integrins, leads to a decrease in the number of autophagosomes in cells on soft substrata. Our data suggest that the stiffness of the microenvironment regulates dormancy and autophagy of breast cancer cells, which could facilitate the long-term survival of metastases in the soft microenvironments at secondary sites.

P1168/B295

Study of VEGF-A Regulation in Osteosarcoma Spheroids Under Various Cellular Stresses Using Microfluidic Devices.

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Three-dimensional (3-D) cell culture provides a convenient micro-environment to study tumor biology and their survival mechanisms[1]. Cellular stresses like serum starvation and drug therapy often result in aggressive pro-angiogenetic behavior in tumors. Recent drug studies have shown resistance to chemotherapy by hypoxia inducible factor (HIF) regulated pathways, particularly in osteosarcomas, and most studies done on 3-D models have shown different results from conventional 2-D monolayer cell cultures[2, 3]. An approach with statistically significant sample size to observe responses of cancer spheroids under cellular stresses is desired. In our work, vascular endothelial growth factor of type a (VEGF-A) of osteosarcoma MG-63 spheroids cultured using microfluidic devices is studied under normal

growth and stress conditions (serum deprivation and HIF inhibition) to investigate their roles in spheroid growth. The microfluidic device is designed to culture 5000 uniformed sized spheroids, and enzyme-linked immunosorbent assay (ELISA) is used for quantitative characterization of VEGF-A secreted from the spheroids. Cell viability assay is also performed to estimate apoptotic cell ratios based on imaging. The results are compared to conventional 2-D cell culture ones. The results show that the spheroids cultured for a 12-day time period have specific log phase (~6 days) denoted by increase in size by 12-14% and high VEGF-A secretion. This is followed by a stationary phase and decline phase where the VEGF-A concentration lowers gradually and spheroid growth is restricted (3% decrease in size). Our experimental results show that VEGF-A secretion decreases with increase in stress doses (lower serum concentration or higher drug concentration) in 2-D monolayer cell culture. However, in 3-D spheroid cultures, VEGF-A concentration decreases for low stress doses but increases with high stress doses. The VEGF-A regulation in our 3-D models mimics in *vivo* cases of tumor survival and can provide insightful information to investigate tumor angiogenesis. In conclusion, this work provides an efficient, statistically significant method of studying tumor growth kinetics and stress response mechanisms using in *vitro* 3-D cell cultures and cytokine immunoassays. Hence, it also illustrates the significance of 3-D tumor models over conventionally used 2-D models in cancer research. References 1. P. K. Chaudhuri, M. Ebrahimi Warkiani, T. Jing, Kenry and C. T. Lim, an *alyst*, 2016, **141**, 504-524. 2. T. Das, T. K. Maitia and S. Chakraborty, *Integrative Biology*, 2011, **3**, 684-695. 3. L. Galluzzi, T. Yamazaki and G. Kroemer, *Nature Reviews Molecular Cell Biology*, 2018, **19**, 731-745.

P1169/B296

Profilin1 Is an Important Intracellular and Extracellular Mediator of Endothelial-Tumor Cell Crosstalk in Tumor Cell Motility and a Novel Prognostic Biomarker in Clear Cell Renal Cell Carcinoma.

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Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal cancer (found in >75% patients) and associated with poor patient prognosis. Additionally, five-year survival of patients with advanced ccRCC is only 10%. The objective of the study is to test if upregulation of the actin-binding protein profilin-1 (Pfn1) promotes ccRCC progression through a pro-angiogenic tumor microenvironment. We performed bioinformatics analyses of TCGA datasets to demonstrate elevated expression of Pfn1 correlated with clinicopathological features of advanced tumors and shorter overall and disease-free survival of ccRCC patients, but not in other histological subtypes of renal cancer. A hallmark of ccRCC is a vascular-rich tumor microenvironment due to genetic inactivation of von Hippel Lindau leading to dramatic upregulation of angiogenic mediators. Immunohistochemistry of human ccRCC tissue microarray revealed that the vast majority of ccRCC tumors exhibit strong Pfn1 expression in stromal (vascular/lymphocytic) cells rather than in tumor cells, a finding further corroborated by single-cell transcriptome studies demonstrating robust transcriptional upregulation of Pfn1 in endothelial cells (EC) of tumor-associated vasculature. Overexpression and knockdown studies provide evidence for endothelial Pfn1-dependent changes in motility of ccRCC cells through a paracrine mechanism that is consistent with alterations in the secreted levels of several cytokine-like molecules. Interestingly, Pfn1 itself is one of the proteins that is released by EC in the extracellular environment, a finding that is further supported by detectable Pfn1 in the serum of ccRCC patients. Furthermore, extracellular Pfn1 stimulates motility of ccRCC motility suggesting that Pfn1 may also have a previously unrecognized extracellular function. Finally, we demonstrated prominent inhibition of ccRCC cell

migration and proliferation, and angiogenesis by novel small molecule antagonist of Pfn1:actin interaction. Collectively, these findings suggest that Pfn1 is an important mediator of endothelial-tumor cell crosstalk, a novel prognostic biomarker and a potential target for intervention in ccRCC.

P1170/B297

Neuroblastoma Differentiation in *Vivo* Excludes Cranial Tumors.

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Neuroblastoma (NB), the most common cancer in the first year of life, is thought to arise from the neural crest (NC)-derived sympathoadrenal lineage and is found almost exclusively in the trunk. To understand why an early-onset cancer would have such a specific localization, we xenotransplanted human NB cells into discrete NC streams in developing zebrafish embryos. We demonstrate quantitatively that human NB cells previously shown to be incapable of differentiation remain undifferentiated when comigrating posteriorly with native NC cells but, upon comigration into the head, readily differentiate into neurons and exhibit decreased survival. Furthermore, we demonstrate that this *in vivo* differentiation requires retinoic acid and brain-derived neurotrophic factor signaling from the microenvironment and cell autonomous intersectin-1-dependent phosphoinositide 3-kinase-mediated signaling, likely via Akt. Our findings suggest a microenvironment-driven explanation for NB's trunk-biased localization in young patients and highlight the potential of differentiation in promoting NB resolution in *vivo*.

P1171/B298

Neuronal Synaptic Proteins Regulate Cancer-associated Fibroblast Pro-tumor Features in Pancreatic Cancer.

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Currently, only 9% of pancreatic ductal adenocarcinoma (PDAC) patients live beyond 5 years. These poor outcomes are, in part, driven by a distinct fibrous microenvironment known as desmoplasia that comprises over 70% of the cancer mass. However, desmoplasia is a double edge sword; its highly immunosuppressive behavior is tumorigenic, yet its removal leads to unrestricted tumor growth. Thus, understanding PDAC's desmoplasia is crucial for selectively harnessing its natural tumor suppressive functions. Previous research revealed that the pro-tumorigenic function of desmoplasia relies on two key components; cancer-associated fibroblasts (CAFs) and the desmoplastic extracellular matrix (D-ECM). Notably, D-ECM stimulates CAFs to express a pre- and post-synaptic protein pair that is not expressed under healthy physiological conditions. CRISPR-Cas9-mediated knock out of the pre-synaptic protein in patient-derived CAFs significantly altered CAFs' natural ability to secrete inflammatory cytokines but maintained the ability to create an aligned D-ECM; classically considered a feature of poor patient outcome. Mono-cultured KO CAFs maintained expression of the post-synaptic protein, suggesting that the expression and engagement of these neuronal proteins in CAFs may have important roles in stromal maintenance that can contribute to the pro- and anti-tumorigenic effects. Additionally, RNA sequencing data comparing KO CAFs to controls identified a significant decrease in the expression of a protein tyrosine phosphatase (PTP) involved in synaptic stabilization and focal adhesion formation

that was previously shown to bind these upregulated synaptic proteins in neurons. PTP CRISPR-Cas9-mediated knock out cells similarly altered CAF cytokine secretion but disrupted CAF myofibroblastic features; that is, the ability to maintain an aligned D-ECM. How PTP signaling affects CAF D-ECM organization, cytokine secretion, and whether it is associated with synaptic protein-induced signaling are the current future directions of this project.

P1172/B299

Using 3d Spheroids to an alyze the Invasion of Lung Cancer Cells Induced by Fibroblasts Under the Treatment of Cigarette Smoke Extract.

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It was reported that cigarette smoke extract (CSE) may participate in the invasion and metastasis of lung tumors through various mechanisms. Using the conditioned medium of the CSE-treated fibroblast (MRC-5) to treat the lung cancer cells (CL1-0), we found that the invasion capability of lung cancer cells was enhanced in the conventional 2D invasion assay. Western blotting assays showed that the CSE enhanced the expressions of the autophagy-relevant microtubule-associated protein 1 light chain 3 β (LC-3B) and decreased the p62 protein. Therefore we suspected that the CSE could induce autophagy in the fibroblast. We further tested this invasion-enhancement capability of the CSE-treated fibroblast using 3D cellular spheroids. We placed Matrigel® mixed with the culture medium into the wells of an ultra-low-attachment 96-well plate. With a 50% volume concentration of the Matrigel®, the cancer cells aggregated to form a spheroid spontaneously after 24 hours. The cancer cells were labelled with the CellTracker dye such that we could use fluorescence microscopy to observe the invasion of individual cells into the gel. For each spheroid, we quantified the cancer cell invasion in 24 hours using the longest invasion distance measured from the spheroid boundary. We observed that the conditioned media of fibroblasts treated by CSE or rapamycin (as a positive control) could enhance the cancer cell invasion in the 3D cellular spheroids. Interestingly, as we co-cultured fibroblasts with the cancer cell spheroid, we observed the fibroblast-led invasion into the Matrigel®. The invasion distances in the co-culture system were longer than those in the mono-culture spheroids. In summary, we demonstrated that the CSE treatment on fibroblast could raise the invasion capability of lung cancer cells using 3D mono-culture and co-culture spheroids. The effect of CSE could be related to the autophagy in fibroblast.

P1173/B300

***RAI14* Is an Actin Cytoskeleton Interacting Protein That Is Upregulated in Pancreatic Cancer and Promotes Src Kinase Stability, Proliferation and Migration.**

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PDAC (pancreatic ductal adenocarcinoma) is one of the most chemoresistant malignances and recognized as the third leading cause of cancer-related deaths. An underlying cause of PDAC's therapy refractory nature is the highly desmoplastic tumor microenvironment that is enriched for extracellular collagen. In this regard, we previously identified integrin alpha one (ITGA1) as a pseudopodium-enriched

protein (PDE) required for protumorigenic collagen signaling in PDAC. Further analysis of PDE proteins co-enriched with collagens and ITGA1 in PDAC led us to the observation that RAI14 (retinoic acid induced fourteen, an kycorbin or NORPEG) is an actin cytoskeleton interacting protein in PDAC cells. We further reasoned that RAI14 expression may be required for the tumorigenic behavior of PDAC as a downstream regulator of collagen/integrin-cytoskeleton signaling. Interestingly, using siRNA-mediated transient silencing, we demonstrate that RAI14 promotes Src kinase protein stability and fibronectin-mediated PDAC cell migration. In light of recent work demonstrating that fibronectin promotes the proper assembly/processing of collagen fibers, our results suggest a potential role for RAI14 as an upstream regulator of collagen trafficking/deposition. Thus, ongoing work is examining the role of RAI14 in collagen processing in and deposition rom PDAC cells using RNAi and CRISPR/Cas9 depletion/deletion methods. These current and prospective findings have potential to illuminate new mechanisms by which PDAC cells foster a malignant microenvironment to supports their expansion and disease progression.

P1174/B301

Extracellular Vesicles Secreted from Pancreatic Cancer Cells Have Bioactive Quiescin Sulfhydryl Oxidase 1 (QSOX1) That Selectively Interacts with the Tumor Microenvironment.

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Pancreatic ductal adenocarcinoma (PDAC) is a solid tumor malignancy that is characterized by a strong desmoplastic reaction. During cancer progression, the extracellular matrix (ECM) becomes increasingly disorganized as cancer-associated fibroblasts deposit dense ECM proteins (collagens, laminins, fibronectin, etc.) which are then modified by matrix-remodeling proteins (MMPs, lysyl oxidase, etc.). In particular, the secreted enzyme QSOX1 is overexpressed in PDAC. QSOX1 is a disulfide bond catalyst that is implicated in cancer invasion, cell migration, and poorer outcomes in cancer patients; however, the specific interactions of QSOX1 with the tumor microenvironment are poorly understood. Because pancreatic cancer is known to release an abundance of extracellular vesicles (EV), we investigated whether QSOX1 was being released into the extracellular matrix via EVs. In this study, we characterized QSOX1-containing EVs and explored how QSOX1 interacted with different ECM protein identities. To analyze the supernatant, PANC-1 cells were cultured in serum free medium for 24 hours and the media was concentrated via spin column centrifugation or differential ultracentrifugation. To characterize EVs, samples were analyzed by nanoparticle tracking analysis and found to be on average 150-200 nm in diameter. EV protein content was then analyzed via Western blot and were positive for QSOX1 along with the exosome proteins Alix and TSG101, while negative for GM-130 and integrin α5. To investigate binding of QSOX1 to various ECM proteins, the concentrated supernatant was resuspended in PBS and allowed to settle on ECM-coated coverslips (laminin, Matrigel, fibronectin, gelatin, and collagen) for 24 hours. Significantly more QSOX1-positive particles were found on the Matrigel, laminin, and fibronectin coating compared to both gelatin and collagen ($p<0.05$). Interestingly, on Matrigel coatings, there was extensive association between QSOX1 and laminin-rich domains, demonstrating further evidence of selective ECM protein localization. Finally, an enzymatic activity assay showed that the supernatant contained 300 nM of functionally active QSOX1, suggesting more than a passive involvement in pancreatic cancer. In summary, we find that QSOX1 containing EVs preferentially associate with laminin(s) and fibronectin through a previously unexplored binding mechanism. Considering how abundant QSOX1 is in the ECM and its ability to rapidly oxidize proteins, these data suggest that QSOX1 plays an important and unappreciated role in ECM remodeling of the tumor microenvironment.

P1175/B302

Synergistic Metalloproteinase-based Remodeling of Matrix by Tumor and Stromal Cells during Metastatic Invasion.

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The process by which tumor cells mechanically invade through the surrounding stroma into peripheral tissues is an essential component of metastatic dissemination. It is known that matrix metalloproteinase (MMP)-mediated extracellular matrix (ECM) degradation plays an important role in this invasive process, and multiple MMPs have been implicated in metastasis. Defining the contribution and interaction between these MMPs during invasion remains a key interest in the development of targeted anti-metastatic therapies. As both tumor and stromal cells secrete MMPs, the goal of this study was to define the relative contributions between these cell types during matrix remodeling and pancreatic (PDAC) tumor cell invasion. To address this question, we employed multiple different stromal fibroblast populations and cultured PDAC cells and measured the capacity of the tumor cells to degrade a fluorescently-labeled gelatin matrix. Interestingly, co-culture of stromal fibroblasts and PDAC tumor cells results in a marked increase in matrix degradation by tumor cells. Importantly, tumor cells co-cultured with the conditioned medium from stromal fibroblasts exhibited a substantial increase in invadopodial-based matrix degradation, indicating that the fibroblast-mediated increase in matrix degradation is due to a secreted protein. This increase in matrix remodeling also potentiated the capacity of cells to invade through transwell chambers by 30%. Western blot analysis and zymography indicated that stromal fibroblasts, but not PDAC cells, express high levels of pro-MMP2. To test if stromal secretion of MMP2 was acting to stimulate matrix degradation by PDAC cells, MMP2 expression was depleted in stromal fibroblasts using siRNA, and the conditioned media was added to PDAC cells. Loss of MMP2 in the stromal cells prevented the increase in tumor-cell based matrix degradation. MMP2 is known to be activated by MT1-MMP. We hypothesized that the MMP2 secreted by fibroblasts was being activated by MT1-MMP on tumor cells. Indeed, depletion of MT1-MMP in tumor cells by siRNA largely blocked matrix remodeling, even in the presence of stromal cell medium. Consistent with this model, zymography revealed that the pro-MMP2 from the stromal fibroblasts is cleaved and activated following incubation with PDAC tumor cells. In Summary**,** these findings implicate an important interplay between MT1-MMP from tumor cells and MMP2 from fibroblasts as a key component for ECM remodeling and invasion.
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P1176/B303

Mapping in *Vitro* Cellular Interactions between Cancer and Normal Cells Via Synthetic Notch Ligand-Receptor Pairs.

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Understanding how cells move within an organism and what other cells they come into contact with is critical for elucidating fundamental mechanisms of development, organogenesis as well as pathologic processes such as metastasis. Here we show, in *vitro*, a synthetic-Notch ligand/receptor pair (synNotch - Morsut et al. Cell 2016) based approach can be used to detect physical interactions between immortalized human retinal pigment epithelial (hTERT RPE-1) cells and patient derived Adenoid Cystic Carcinoma (ACC) cells or mouse mammary epithelium derived cells that overexpress human c-Myc oncogene in the presence of doxycycline (MTB/TOM). hTERT RPE-1 or ACC or c-Myc overexpressing

MTB/TOM cells, expressing synthetic ligand (outer plasma membrane localized GFP), were co-cultured with hTERT RPE-1 cells, expressing transgenic (i) LoxP-tagBFP-STOP-LoxP-DsRed, (ii) synthetic Notch receptor/transcriptional activator (outer plasma membrane anti-GFP nanobody combined with internal Gal4 transcription factor through a transmembrane domain) and (iii) CRE-recombinase fused to Estrogen receptor (ER-T2). By combining FACS and fluorescence microscopy facilitated scratch assays, we find that cells carrying the receptor component of synNotch system can switch their fluorescent reporter expression from tagBFP to DsRed more than 10 times when co-cultured with ligand carrying cells in the presence of estrogen receptor modulator, 4-Hydroxytamoxifen (4-OHT) in comparison to mock control. We also find that it takes about one cell division to switch the fluorescent reporter color of a single, receptor carrying cell after its attachment with a ligand carrying one is achieved. Surprisingly, we observed that ligands are not fixed in position between two interacting cells but also are taken in by the receptor carrying cells via a trogocytosis-like process. Our findings suggest that dynamic physical interactions between cells can be mapped using cell surface localized synthetic Notch ligand/receptor pairs in a highly specific manner. Ultimately, we seek to integrate our system into mice to study how an individual cancer cell metastasizes and identify all of the nontumor host cells with which it comes into contact during the metastatic process. We envision the same sensor mice will be valuable to study other processes, such as neurogenesis or development of the immune system.

P1177/B304

Secretome of Human Breast Cancer Cells Induces Changes in Gene Expression Profile but It Is Not Involved Transformation of Mesenchymal Stem Cells from Wharton´s Jelly.

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Mesenchymal cells are cells that have great potential for differentiation, proliferation and self-renewal, they are present in all tissues. Mesenchymal stem/stromal cells (MSCs) are commonly used as a source of cellular therapy due to their strong immunosuppressive and regenerative effects. Despite the relevance and potential impact of WJ-MSCs on regenerative medicine and tissue remodeling, there is a posibility that the use of these cells in a non-diagnostic procarcinigenic environment on patients with cell therapy this will be considered a risk to tumour transformation, which remains in controversy still. Therefore, a study model is needed to investigate whether tumoral secretome influences on the molecular profile of WJ-MSCs indicating tumour transformation. So the above was investigated in an in *vitro* model. Methods: Secretome was obtained at 24 and 48 h from MCF-7 cultures and it was added on WJ-MSCs and incubated during 48 h. Epifluorescence Microscopy was used to observe changes in cell morphology and citoskeleton organization using phaloidin staining. Finally gene expression was evaluated by RT-PCR for different genes involved in proliferation, immunologic profile as well as mesenchymal markers. Results showed that umbilical cord-derived WJ-MSCs accomplished the criteria proposed by International Society for Cell Therapy (ISCT). Secretome from MCF-7 induced atypical proliferative but not exacerbated behavior of WJ-MSCs in comparison with cells without stimuli. In addition, we observed that cytoskeleton of mesenchymal stem cells changed when the cultures were in presence of secretome of cancer cells. Gene expression of molecules involved in immunological regulation also changed, but genes related to tumoral transformation or excessive proliferation were not affected when WJ-MSCs were incubated with secretome. We concluded that WJ-MSCs are not capable to present characteristics of malignant cells in presence of tumoral environment. It is necessary

to continue with more investigations for ensure that MSCs does not change to tumoral form; nevertheless this work show evidence that cell therapy with MSCs administration be sure and no represent risk for tumoral transformation.

P1178/B305

High-throughput Microfluidic Platform for in Vitro Vascularization of Human Organoids and Tissue Explants - Cell Cultures' Missing Component?

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Vasculature is a crucial ingredient of human organs and tissues. In addition to being a vehicle for blood circulation, exchange of angiocrine factors contribute to tissue function and development. The challenge in creating better biomimetic models lies in capturing the 3D morphology, heterogeneity and boundary aspects of tissues. Microtissues such as spheroids or organoids show often limited growth potential and an immature phenotype due to the lack of circulation. One way to improve vascularization of organoids or explants is by transplanting them into a host (*i.e.* mouse). However, the low predictivity of animal models, boost the development of in *vitro* alternative strategies. Microfluidic techniques are increasingly recognized as an important toolbox able to add physiologically relevant cues to traditional cell culture models. Here, we introduce a microfluidic platform that is capable of vascularization of tissues such as organoids, spheroids and tissue fragments. The platform comprises 64 independent microfluidic chips arranged in a microtiter plate format. Each chip comprises an extracellular matrix gel that is patterned by means of a surface tension technique called PhaseGuiding. In each chip two blood vessels are grown on lateral side of the free-standing ECM gel. A gradient of angiogenic factors is applied to induce vascular bed formation. Tissues are grafted onto the vascular bed by placing it on top of the ECM gel, containing the vascular bed. Tissue dependent vessels remodeling and stabilization can be monitored overtime by real time imaging and barrier integrity. When liver spheroids or organoids are used, vessels became stabilized and fully leaktight in the system. Moreover, the expression of $CD31^+$ cells around and in within the spheroids proves that endothelial cells migration and tissue envelopment occurred during co-culture. The high number of units (up to 64 chips in 384 well format) enables functionality studies and compound screening in a robust and automated way. We propose the use of the Organoplate® Graft as a vessels grafting platform for multiple 3D tissues allowing drug discovery and disease modeling in a more physiological environment. The method provides an in vitro alternative to current xenograft techniques and may fill up a crucial gap in current day cell culture.

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Characterization of Reprogrammed Human Cancer Cells Contributing to Tumor-free Chimeric Mammary Gland Outgrowths.

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Cellular behavior can be influenced by local tissue microenvironment. When transplanted on their own, human embryonal carcinoma (Ntera2) cells form tumors. However, the tumorigenic phenotype is lost upon mixing and co-inoculation with normal mouse mammary epithelium into epithelium-divested mammary fat pads of Athymic nude mice. Despite loss of tumorigenic phenotype, we have shown that

tumor cells persist throughout at least four transplant generations, indicating that the human cells are self-renewing and respond to in *vivo* signals during mouse mammary gland redevelopment. This phenotypic shift from tumorigenic to non-tumorigenic shows the normal mammary epithelium and microenvironment are able to reprogram the human carcinoma cells. It is predicted that these differences in phenotype can be explained via altered gene expression patterns. Magnetic cell sorting revealed the reprogrammed cells are tightly associated with mammary epithelium because there was contamination of the cell populations. In order to study the reprogrammed human cells individually, single cell RNA sequencing was used. Transcriptional differences between the normal human and mouse chimeras versus tumorigenic human cells were compared. Identifying changes in gene expression will narrow down important contributors to the normal phenotype, thereby gaining greater insight into the prevention of cancer.

P1180/B307

Extracellular Vesicles from Breast Cancer Cells Stimulated with Linoleic Acid Promote an giogenesis in Human Endothelial Cells.

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Breast cancer is the leading cause of death for women worldwide maintaining upward trends in incidence and mortality. Several studies have established a clear association between obesity, a high-fat diet and the development of breast cancer. One of the most abundant fatty acids in the diet is linoleic acid (LA), that can be used as an energy source and it is the ligand for two G protein coupled receptors (FFAR1 and FFAR4) and their activation induces several biological processes including proliferation, migration and invasion. On the other hand, during cellular progression, cellular communication is a key event, one mechanism of communication is mediated by small spherical fragments of membranes called extracellular vesicles (EVs). The EVs are secreted by normal and malignant cells, their function is variable and depends on the type of charge that they carry on, as well as the cell type from which they originated. A variety of reports demonstrates that EVs mediate various biological processes associated with cancer progression, including tumor angiogenesis. In this study, we demonstrate that EVs isolated from supernatants of MDA-MB-231 cells stimulated with LA increase the number of secreted EVs. In addition, the EVs from MDA-MB-231 cells stimulated with LA induce migration, proliferation, invasion, secretion of MMP-2 and MMP-9 and angiogenesis in HUVEC cells. This work was supported for a grant from CONACYT-MEXICO (255429) A. G-H is supported for a predoctoral training grant from CONACYT-MEXICO

P1181/B308

Analysis of Early Cellular and Sub-cellular Changes during Tumor Initiation and Progression in Live an imals.

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Head and neck squamous cell carcinoma (HNSCC), most of which arise in the oral cavity, is one of the most common cancers with a 65 % 5-year survival rate. It accounts for more than 650,000 cases and 330,000 deaths worldwide annually. In 2019, it is estimated to have more than 53,000 new cases of HNSCC in the United States, which consists of 3% of all new cancer cases. Understanding the mechanisms that control initiation, progression, and spreading of the tumor to distal sites is of

paramount importance to develop effective therapies. To this end, we have used a well-established carcinogen model in live mice that mimics the progression of HNSCC observed in patients. Here, we show, for the first time, the visualization within the same animal of the onset and progression of cancerous and pre-cancerous lesions that were followed for 22 weeks. By using intravital subcellular microscopy (ISMic), we acquired information on cellular and subcellular changes in the tumors cell and the microenvironment (blood vessels, ECM, an immune cells), and specifically, we focused on changes in the architecture of the actomyosin cytoskeleton, mitochondrial metabolism, and autophagy. This powerful approach allows for the first time to investigate the cell biology of tumor initiation and progression in a live animal.

P1182/B309

Stromal Matrix Metalloproteinase 3 (mmp3) Inhibits Breast Cancer but Is Required for Metastasis from the Primary Tumor.

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Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that regulate the tissue microenvironment. MMP3 regulates both breast development and cancer progression and localizes to both mammary epithelial and stromal cells in normal and tumor mouse mammary tissues. Epithelial MMP3 overexpression promotes mammary tumor development. However, the functions of stromal and epithelial MMP3 have not been distinguished. We investigated the requirements for stromal MMP3 during breast cancer. We transplanted PyMT cancer cells, which express MMP3, into mammary glands of mice expressing (MMP3 Het) or not expressing (MMP3 KO) MMP3. Surprisingly, tumor burden increased in MMP3 KO mice, suggesting an inhibitory role for stromal MMP3. We next analyzed proliferation, apoptosis, and immune cell infiltration by immunohistochemistry to identify stromal MMP3's contribution to tumor progression. Tumors from KO recipient tumors had decreased cell proliferation marker phospho-histone H3 and neutrophil recruitment but increased $CD4^+$ and $CD8^+$ T cell infiltration compared to Het tumors. Apoptosis was not significantly different. Subsequent bone marrow transplantation experiments confirmed an inhibitory role for a stromal MMP3, but MMP3-deficient bone marrow, $CD11b^+$ cells, and CAFs were insufficient to increase tumor burden, and bone marrow-derived MMP3 even had a tumor promoting role. We next investigated stromal MMP3's role during lung metastasis. In contrast to the inhibitory role for stromal MMP3 in the primary tumors, stromal MMP3 was required for lung metastasis by orthotopic transplantation of breast cancer cells. Interestingly, stromal MMP3 inhibited lung metastasis by tail vein injections of breast cancer cells, suggesting that MMP3 impacts metastasis by effects at the primary tumor site. Together, this study suggests that stromal MMP3 has both protective and tumor promoting roles during breast cancer and highlights the context-specific functions of MMP3 during breast cancer.

P1183/B310

Anti-inflammatory and Antiangiogenic Therapies Inhibit Endothelial-to-mesenchymal Transition in TRAMP Mice Prostate.

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Prostate cancer is the most frequently diagnosed cancer among men in about half of the countries worldwide. Reactive stroma comprises a set of changes in the stromal compartment, including stimulated angiogenesis and inflammatory cell influx, which lead to prostate cancer progression and metastasis. Although it is well known that cells in reactive stroma have a mesenchymal secretory phenotype, their originating mechanisms from adjacent tissue are still poorly understood. Thus, the aim herewith is to investigate whether reactive stroma cells can derive from resident endothelial cells and the influence of inflammatory and angiogenic inhibitors on this endothelial-to-mesenchymal transition (EndMT). Male Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) mice (12 week-old) were divided into the groups: Control (TRCON): received the vehicles used for drug dilution; Celecoxib (TRCEL): received celecoxib (15 mg/kg), an anti-inflammatory drug; Nintedanib (TRNTB), received nintedanib (10 mg/kg), a triple angiokinase inhibitor; Nintedanib + Celecoxib (TRNTCEL): received the combination of treatments. After 6 weeks, dorsolateral prostate samples were collected for morphological, immunohistochemical and Western Blotting analyses. Nintedanib treatment resulted in decreased incidence of well-differentiated adenocarcinoma (PC) foci in relation to control group, regardless of celecoxib co-administration. This finding was concomitant with reduced percentage of PCNA immunolabeling in both TRNTB and TRNTCEL groups. TGF-β1 and TGF-β2, which have recognized roles in driving reactive stroma development and EndMT, respectively, showed lower protein levels following combined treatment, whereas vimentin, a well-known mesenchymal cell marker, had reduced expression in all treated groups. Microvessel density (MVD) was significantly decreased in the TRNTCEL group, as demonstrated by CD31-positive vessel count, as well as the protein levels of Snail, a transcription factor that mediates EndMT. Nintedanib is an effective antitumor agent against prostate cancer progression in TRAMP mice. Moreover, its association with the anti-inflammatory drug celecoxib results in additional beneficial effects towards glandular balance and tumorigenesis delay, such as lowered MVD and TGF-β1 protein levels. At the same time, decreased vimentin, TGF-β2 and Snail protein expressions in the TRNTCEL group suggest that EndMT is indeed a source of activated stromal cells in prostate cancer and that inhibiting angiogenesis and inflammation in this stromal milieu may be a promising strategy to restrict this cellular recruitment. Financial support: State of São Paulo Research Foundation (2018/16299-9).

P1184/B311

Evix Elisa Tools Reveal Differences in Cd3, Cd4, Cd8 and Mhc Class 2 Positive Extracellular Vesicles (evs) Derived from the Colon Cancer Cell Lines Sw480 and Sw620.

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SW480 and SW620 are paired cell lines established from a primary tumor and its metastatic counterpart obtained from the same patient and have a shared genetic background. These cell lines are considered to be a useful model for the changes which may take place during progression of colon cancer. Thus, the SW480 and SW620 cell pairing provides insight into metastatic and malignant behavior of human colon cancer. We report that using EVix Elisa tools we can identify differences between extracellular vesicles (EVs) released by these respective cell lines. EVs from SW480 cells are distinct from EVs from SW620 cells. While both SW480 and SW620 have similar tetraspan expression patterns on their respective EV populations, a marked difference exists in immune associated molecules. We have found that EVs from SW480 cells can be captured with antibodies against CD3, CD4, CD8, MHC Class 1 and 2 and TCR CB1. These captured EVs were detected by anti-tetraspan (CD9, CD63, CD81) antibodies. In contrast EVs from SW620 cells were not captured using the same antibodies against CD3, CD8, TCR CB1 and MHC Class 2.

EVs from SW620 were positive for CD4 and MHC Class 1 antigens similar to EVs from SW480. Comparable amounts of starting EVs from the SW480 and SW620 cell lines were evaluated as determined by Cholera Toxin B capture and human tetraspan (CD9, CD63, CD81) detection. The presence of immune associated molecules in EVs isolated from human SW480 and SW620 colon cancer cell lines is of interest. The differences in the EV populations positive or negative for immune associated molecules suggest differences in respective tumor cell interaction with the host immune system.

P1185/B312

Analysis of Oncogenic Exosomal Micro-RNAs Released from Triple Negative Breast Cancer and Cancer Stem Cells.

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IntroductionTriple-negative breast cancers (TNBCs) are defined by lack of estrogen receptor, progesterone receptor and human epidermal growth factor receptor type 2 (HER). TNBC comprise 15% of all breast carcinomas and have high rates of recurrences and mortality. TNBC is characterized by an abundance of treatment-resistant breast cancer stem cells (CSCs) and very poor prognosis. Since targeting these CSCs may improve treatment outcome of TNBC, it is imperative to investigate new therapeutic approaches. Exosomes have emerged as critical mediators of intercellular communication in tumor microenvironment. Exosome-secreted miRNAs have been shown to induce a number of biological functions including tumorigenesis. Cancer cells modify their microenvironment by transferring miRNAs that are suitable for tumor growth and progression through exosomes. MicroRNAs are specific to various physiological and pathological conditions thus exosomal miRNAs offer great hope for the diagnosis, prognosis and possibly prediction of cancer progression. This study was sought to analyze oncogenic exosomal miRNAs secreted by TNBC and TNBC stem cells and their possible role in tumor diagnosis/ prognosis. **Methods** Exosomes were isolated from TNBC and TNBC stem cells' serum depleted conditioned media. Exosomes were characterized by structural analysis (TEM, AFM), protein markers (CD63, CD81), Immunofluorescence and quantitative real time PCR. TNBC and TNBCSC secreted exosomal miRNAs were isolated and subsequently evaluated by RT-qPCR. **Results**Our results indicate significant upregulation of target exosomal miRNAs in TNBC cell lines (MDA MB 231, MDA MB 468) in comparison to normal breast epithelial cells (MCF10A). In addition, some previously unreported exosomal miRNAs were found to be highly expressed in TNBC stem cells. **Conclusion** These preliminary results demonstrate a significantly higher expression of exosomal miRNAs in TNBC stem cells which may serve as novel diagnostic/ prognostic biomarkers.

P1186/B313

Evaluation of Anticancer Drugs on Human Lung 3D Spheroids.

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Non-small cell lung cancer (NSCLC) represents 85% of all lung cancers. A549 is the one of the most common NSCLC cell line used for modelling alveolar region of lung cells. The goal of this study is to grow A549 lung cancer cells in 3-dimensional spheroid form in *vitro to* mimic tumor like conditions in lung cancer. The main features of spheroids that are similar to tumors include their structure, assembly, hypoxic conditions, and nutrient gradients. Anti-cancer drugs, Crizotinib and Erlotinib were added to determine cellular response of lung cells in 3D spheroid conditions. Both the drugs have been approved

by the FDA in USA as First-Line lung cancer therapy for specific patients. We aim to utilize this model to compare the efficacy of these chemotherapeutic agents on A549 cells in 2D monolayer versus 3D spheroid models.

P1187/B314

Microenvironmental Stress and the Cell Nucleus: an Essential Conversation for Breast Cancer Progression.

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Oxidative stress (OS) in the tumor microenvironment resulting from an imbalance in reactive oxygen species is involved in cancer progression; yet its impact on chromatin and the epigenome known to drive cancer phenotypes remains obscure. We have identified that the nuclear mitotic apparatus protein (NuMA), a chromatin organizer, interacts with transcriptional coactivator, lens epithelium derived growth factor (LEDGF) and remodeling and spacing factor (RSF-1) protein that under OS is overexpressed and is associated with H3K9me2, a known suppressor of LEDGF. **Our central hypothesis is that NuMA-LEDGF-RSF1 interaction controls cancer progression under stress by influencing transcriptional regulation.** We use the HMT-3522 model of triple negative breast cancer progression that in 3D cell culture mimics the phenotypically ductal carcinoma in-situ (S2 cells) and invasive ductal carcinoma (T4-2 cells). Acute OS has been induced with exposure to 250 µM H_2O_2 for four hours. NuMA, LEDGF and RSF-1 show lower expression in T4-2 cells compared to S2 cells. However, prolonged exposure to ROS (25 µM H_20_2 for four weeks) results in a loss of NuMA and LEDGF and increased RSF1 expression in S2 cells, hence mimicking the changes observed in *vivo*. In addition, the size of the nucleus increases in NuMA silenced S2 cells but not in those silenced for LEDGF under OS with no observable changes in nuclear circularity. These results highlight the need to investigate the role of NuMA and LEDGF in response to oxidative stress in cancer progression. To further understand the role of NuMA under chronic OS in influencing cancer cell phenotype, we will explore NuMA silencing using CRISPR in S2 cells. Findings from this study will provide insight to investigate possible therapeutic targets for triple negative breast cancer.

P1188/B315

Nos2 Modulates Hypoxic Gradients in an in *Vitro* Model of the Tumor Microenvironment Based on Restricted Diffusive Exchange.

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The solid tumor microenvironment (TME) is heterogenous, and cellular phenotypic variation within it both drives and arises in response to varying concentrations of soluble molecules. In tumors, cellular consumption of soluble molecules (such as $O_{2\ and}$ nutrients) diffusing from capillaries generates concentration gradients of these molecules. As a tumor grows, the tumor core becomes hypoxic, nutrient deprived, and eventually necrotic. The spatial distribution of phenotypic variation within the TME is poorly understood and is difficult to study with standard 2D cell culture (which lacks physiological

complexity) or spheroids (which are highly variable structures with interiors not easily accessible to live-cell optical microscopy). We utilized a radially symmetric 2D chamber system which restricts the exchange of soluble molecules to model the cell-generated $O_2$and nutrient gradients within the TME in *vitro to* investigate the role these gradients on tumor and immune cell phenotypes, using mouse mammary tumor cells (4T1) and mouse macrophages (ANA-1) individually and in coculture. 4T1 syngeneic mouse tumors model human triple negative breast cancer (TNBC). Macrophages are the most abundant nonneoplastic cell in the TME, have been shown to localize to hypoxic/necrotic regions within tumors, and play an immunosuppressive role in tumorigenesis. We demonstrated that 4T1 cells cultured in this system form O_2 gradients within 2 hours using spatially resolved, ratiometric measurements of dissolved O_2 concentration. Gradients of intracellular hypoxia were verified through immunofluorescence imaging of reduced pimonidazole and nuclear localization of hypoxia-inducible factor 1α (Hif1α). Within 60 hours, 4T1 cells in the chambers migrated away from hypoxic regions and formed stable disks ~1 mm in diameter around the O_2 source. Beyond the disk, cells either detach from the surface or die, analogous to necrotic zones in tumor tissue. We applied this system to investigate the organization of nitric oxide synthase 2 (Nos2) and cyclooxygenase-2 (Cox2) expression within the TME. Nos2 and Cos2 interact in a proinflammatory feed-forward loop, and in TNBC their co-expression is a very strong predictor of poor prognosis. Using multiplexed immunofluorescence labeling, we showed that Nos2 and Cox2 were expressed in spatially distinct environments, with Nos2 expression localized in hypoxic regions. These results were analogous to those observed in spheroids and xenograft tissue sections, and they suggest that the Nos2 / Cox2 feed-forward loop occurs through an paracrine mechanism. We further showed that ANA1 macrophages generated hypoxic gradients and that ANA1s stimulated with inflammatory cytokines (IFNg+LPS) modulated the magnitude and extent of the hypoxic gradient.

P1189/B316

Exploring the Role of Stroma in Breast Cancer Using a Microfluidic 3D Mammary Duct Platform.

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It has been shown that cross-talk occurring between the epithelial cells and fibroblasts of mammaliantissue can play a role in the development of breast cancer. The purpose of our research is to understand the role this communication plays in the formation and proliferation of mammary carcinomas with respect to the transmembrane heparan-sulfate proteoglycan, syndecan-1 (sdc-1). In clinical studies of tissue biopsies from breast cancer patients, it has been shown that sdc-1 is actively expressed and shed in tumor-associated fibroblasts. In addition, published studies indicate that sdc-1 ectodomain molecules can play an important role as a co-receptor for FGF receptor. Based upon this information, we are exploring the role that sdc-1 ectodomain-based cross-talk between epithelial cells and fibroblasts may play in promoting epithelial cell tumorigenesis by increasing FGF-based cell proliferation. In previous work, we have shown that NIH-3T3 fibroblast cells actively express and shed sdc-1 ectodomain in culture, and have demonstrated an increase in proliferation of mammary epithelial cells co-cultured with NIH-3T3 cells. We are now expanding these studies to a more physiologically-relevant system by adapting our existing microfluidic 3D mammary duct platform to allow the inclusion of stromal fibroblasts. 3D culturing of fibroblasts in the presence of epithelial ductal structures in the chip has been successful, and we are now initiating experiments utilizing modified fibroblasts to directly test the role of shed sdc-1 molecules in the initiation of epithelial tumor formation.

P1190/B317

Cancer-associated Mutations That Inhibit Phosphofructokinase-1 Activity Confer Increased Resistance to Reactive Oxygen Species Stress.

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Cancer cells have an altered metabolic program, termed aerobic glycolysis or the Warburg effect, to provide energy and building blocks required to support rapid proliferation. The glycolytic "gatekeeper" enzyme phosphofructokinase-1 (PFK1), which catalyzes the step committing glucose to breakdown, is dysregulated in cancers. While increased expression and activity of PFK1 has been demonstrated in cancers, how somatic mutations affect metabolic adaptation is incompletely understood. We have previously mapped 44 cancer-associated somatic mutations in the platelet isoform of PFK1 (PFKP) and found that the majority were predicted to inhibit enzymatic activity. We characterized one of these mutations, PFKP-D564N, to gain insight into how inhibitory PFK1 mutations can contribute to cancer cell metabolism. Purified, recombinant PFKP-D564N had decreased affinity for the sugar substrate fructose 6-phosphate and decreased maximal catalytic activity compared to the wild type enzyme. We determined the crystal structure of PFKP-D564N to 3.4Å resolution to determine how the mutation inhibited enzymatic activity. Only small changes in structure were observed compared to wild type PFKP. However, the mutation was predicted to disrupt a salt bridge between aspartic acid 564 and arginine 319, which are evolutionarily conserved from bacteria to human. Cancer-associated mutations of this Asp-Arg pair were also identified in the liver isoform PFKL. Recombinant PFKL-R310Q and PFKL-D553N had decreased affinity for fructose 6-phosphate and decreased maximal catalytic activity, consistent with our observation on PFKP activity. PFKP-D564N was stably expressed in MTLn3 rat mammary adenocarcinoma cells to determine if expression alters cancer cell metabolism. Cells expressing PFKP-D564N displayed decreased flux of glucose to lactate compared to control cells or cells expressing wild type PFKP. Inhibition of glycolytic flux by loss of function mutations may confer a selective advantage for cancer cell growth and metastasis by redirecting carbon flow through the pentose phosphate pathway. To test this hypothesis, we assessed survival after a reactive oxygen species (ROS) stress. Cells expressing PFKP-D564N were resistant to ROS-induced death, as they showed a significant increase in the number of live cells after treated with 1µM paraquat for twenty-four hours compared to control cells and cells expressing wild type PFKP. Taken together, our results suggest inhibitory PFK1 mutations can alter cancer cell metabolism and provide resistance to ROS.

Cancer Therapy 2

P1191/B318

A Fatty Acid Oxidation-dependent Metabolic Shift Regulates the Adaptation of *BRAF*-mutated Melanoma to MAPK Inhibitors.

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Treatment of *BRAF*^{V600E}-mutant melanomas with mitogen-activated protein kinase inhibitors (MAPKi) results in significant tumor regression, but acquired resistance is pervasive. To understand non-mutational mechanisms underlying the adaptation to MAPKi and to identify novel vulnerabilities of melanomas treated with MAPKi, we focused on the initial response phase during treatment with MAPKi. By screening proteins expressed on the cell surface of melanoma cells, we identified the fatty acid transporter CD36 as the most consistently upregulated protein upon short-term treatment with MAPKi. We further investigated the effects of MAPKi on fatty acid metabolism using in vitro and in vivo models and analyzing patients' pre- and on-treatment tumor specimens. Melanoma cells treated with MAPKi displayed increased levels of CD36 and of peroxisome proliferator-activated receptor α (PPARα)-mediated and carnitine palmitoyltransferase 1A (CPT1A)-dependent fatty acid oxidation (FAO). While CD36 is a useful marker of melanoma cells during adaptation and drug-tolerant phases, the upregulation of CD36 is not functionally involved in FAO changes that characterize MAPKi-treated cells. Increased FAO is required for *BRAF*^{V600E}-mutant melanoma cells to survive under the MAPKi-induced metabolic stress prior to acquiring drug resistance. The upfront and concomitant inhibition of FAO, glycolysis and MAPK synergistically inhibits tumor cell growth in *vitro* and in *vivo*. Thus, we identified a clinically relevant therapeutic approach that has the potential to improve initial responses and to delay acquired drug resistance of BRAF^{V600E}-mutant melanoma.

P1192/B319

Comparative Proteome an alysis of Breast Cancer Tissues to Investigate Breast Cancer Metabolism.

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Breast cancer (BC)-associated deaths among women are dramatically increasing. One of the reasons for the increase is the absence of an easy to perform early-diagnosis test, which would allow physicians to take timely precautions to prevent the growth of the tumor tissue. Unfortunately, however, there is no easy to use, affordable, specific and sensitive laboratory test for BC diagnosis. In this study, we aimed to discover potential diagnostic or prognostic biomarkers by investigating the global changes occurring in protein profiles of BC tissues. For this purpose, BC tumor tissues and their corresponding healthy counterparts were collected, subtyped, and subjected to comparative proteomics analyses using two-dimensional gel electrophoresis (2-DE) and difference gel electrophoresis (DIGE) coupled to matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/TOF). Western blot analysis was used to verify changes occurring at the protein levels. Bioinformatics analyses performed with differentially regulated proteins highlighted the changes occurring in proteins, namely glycerol-3-phosphate dehydrogenase 1 (GPD1) and monoacylglycerol lipase (MAGL) and directed our attention to triacylglyceride (TAG) metabolism. These proteins were down-regulated in tumor groups in comparison to the control group. GPD1 and MAGL might be promising tissue-based protein biomarkers with a predictive potential for BC.

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P1193/B320

Targeting Cancer Metabolism in BRCA Deficient Breast Cancer.

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Women carrying a single defective copy of BRCA1 or BRCA2 are at dramatically increased risk of developing breast and ovarian cancer. Specifically, BRCA-deficient ($BRCA^{def}$) breast cancer is associated with early onset, high grade, rapid growth, recurrence and metastasis. There is a pressing need for new approaches to prevent, detect, and treat breast and other cancers in women carrying BRCA mutations. The role for the BRCA1 and 2 gene products in homologous recombination (HR)-mediated double strand break (DSB) repair provides a rationale for the well-known "synthetic lethality" strategy of blocking other forms of DNA repair such as non-homologous end-joining (NHEJ) DSB repair as a means to differentially sensitize $BRCA^{def}$ breast cancer cells to DNA damage. Recent studies from the Kron Lab describing how cancer metabolic reprogramming can modulate the hexasomine biosynthetic pathway (HBP) to enhance NHEJ repair suggest a mechanism for how $BRCA^{def}$ cancer cells may compensate for their HR repair deficiency, offering a new opportunity for synthetic lethal targeting. The HBP generates the activated sugar UDP-GlcNAc, allowing transfer of GlcNAc moieties to protein serine and threonine residues by the enzyme O-GlcNAc transferase (OGT). Subsequent removal of O-linked GlcNAc from proteins is mediated by O-GlcNAcase (OGA). As proof-of-principle, the Kron Lab has examined the impact of modulating O-GlcNAcylation on response to genotoxic therapy in breast cancer. Using xenograft tumors formed from the MCF-7 breast cancer cell lines, we found that blocking O-GlcNAcylation via knockdown of OGT or treatment with the OGT inhibitor alloxan conferred radiation sensitivity. In turn, knockdown of OGA or treatment with the OGA inhibitor PUGNAc to promote O-GlcNAcylation conferred radiation resistance. To explore the hypothesis that O-GlcNAcylation might be critical for cells to survive genomic stress in cells lacking BRCA genes, we examined transient knockdown of OGT or OGA in the $BRCA^{def}$ breast cancer cell line MD-MBA-436 using inducible shRNA miRs. When $BRCA^{def}$ cells were irradiated after knockdown of OGT, cells displayed persistent yH2AX foci, indicating an enhanced DNA repair defect. Similarly, in mice bearing MD-MBA-436 tumors exposed to radiation while being treated with alloxan to block OGT, the tissue displayed persistent yH2AX immunoreactivity and decreased Ki67 levels, indicating a prolonged DNA damage response. Our data to date confirm that targeting the HBP sensitizes $BRCA^{def}$ cells to radiation in *vitro* and in *vivo*, suggesting potential as a target for synthetic lethality and/or sensitization to genotoxic cancer therapy in patients.

P1194/B321

AMPK Activators Suppress Cancer Cell Metabolism and Growth Both in *Vitro* and in *Vivo* Providing a Novel Therapy.

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AMP-activated protein kinase (AMPK) plays an important role in the regulation of cellular energy homeostasis. Metformin (biguanide) is a known AMPK activator used as a therapeutic agent for the treatment of Type-II diabetes. In the present study, Metformin, and AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) were used as AMPK activators to monitor tumorigenesis and suppression of cancer cells. The cytotoxicity of Metformin and AICAR were determined in both human pancreatic (PANC-1) and human mammary carcinoma cells (MDA-MB 231), respectively. The results showed that both Metformin and AICAR significantly inhibit cell proliferation in a dose and time-dependent manner. However, the IC-50 of Metformin was slightly higher as compared to AICAR. Furthermore, *in-vivo*

models were established through xenograft tumor models in athymic nude mice by injecting PANC-1 and MDA-MB 231 cells subcutaneously (5x 10^6) suspended in matrigel into the right flank. When tumor size was between 500-1000 mm^3, mice were treated with Metformin and AICAR intraperitoneally at three different doses (100, 200, 400 mg/kg). The tumor size and weight were measured. Metformin and AICAR showed to significantly inhibit tumor growth (size and weight) after 21 days of treatment. However, no significant differences were observed with the low dose. This study demonstrates that AMPK activators, Metformin and AICAR, possess anti-tumor activity on human pancreatic (PANC-1) and breast cancer (MDA-MB 231) cell lines. These results strongly suggest that the activation of AMPK can be a novel anti-cancer therapy.

P1195/B322

Defective Glycan Branching Compromises the Activity of the Novel Immunotherapy Target CD73 in Human Liver Cancer.

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CD73 is a ubiquitous ecto-enzyme that converts adenosine 5'-monophosphate (AMP) to adenosine. Several anti-CD73 therapeutic antibodies are undergoing clinical development because extracellular adenosine promotes tumor growth and metastasis via its immunosuppressive activity. On the other hand, CD73-generated adenosine is critical for protecting the epithelial barrier, thereby functioning in an anti-tumor fashion. Understanding how CD73 activity is regulated in normal versus tumor cells can yield novel insights into the functional complexity of this important cancer target. The objective of this study was to characterize CD73 regulation in normal liver and hepatocellular carcinoma (HCC), the most common form of primary liver cancer. CD73 is a complex molecule that undergoes *N*-linked glycosylation, disulfide bond-mediated homo-dimerization, and membrane association via a glycosylphosphatidylinositol (GPI) anchor. Previously we identified a novel splice variant isoform of CD73 (CD73S) that is catalytically inactive and selectively expressed in HCC, alongside canonical CD73. In this study, we examined canonical CD73 expression, sub-cellular localization, and enzymatic activity using molecular, biochemical, and cellular analyses on human HCC surgical specimens (N=23 adjacent liver-tumor pairs), coupled with mechanistic studies in HCC cells. We analyzed site-specific CD73 glycan signatures in normal liver and HCC tumor tissue via mass spectrometry glycomics analysis. CD73 was primarily expressed on tumor hepatocytes, where it exhibited cytoplasmic distribution and abnormal *N*-linked glycosylation, independent of HCC etiology, tumor stage, or fibrosis presence. Aberrant glycosylation of tumor-associated CD73 resulted in a 3-fold decrease in 5´-nucleotidase activity ($p<0.0001$). Biochemically, tumor-associated CD73 was enriched in high mannose, but deficient in hybrid and complex glycans specifically on residues N311 and N333, located in the C-terminal catalytic domain. Blocking N311/N333 glycosylation via site-directed mutagenesis produced CD73 with significantly decreased 5´-nucleotidase activity in *vitro*, similar to the primary tumors. Glycosylation-impaired CD73 partially co-localized with the Golgi structural protein GM130, which was strongly induced in HCC tumors. In conclusion, we provide the first detailed characterization of CD73 glycosylation in normal and tumor cells, revealing a novel mechanism leading to the functional suppression of CD73 in human HCC tumors. The present findings have translational implications for therapeutic candidate antibodies

targeting cell-surface CD73 in solid tumors and small molecule adenosine receptor agonists that are currently in clinical development for HCC patients.

P1196/B323

High Throughput CAR-T Cell Potency Assay with Single Cell Resolution.

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Adoptive cellular cancer therapies, especially the usage of autologous T cells expressing engineered antigen receptors (CAR-T cells) represent a promising new cancer therapy tool. The evaluation of quality, specificity and killing efficiency (potency) of CAR-T cell populations is crucial for the development of potent and safe patient specific CAR-T cell products. Potency usually is evaluated on the population level by measurement of Interferon-gamma secretion or Cytotoxic T-cell assays, such as ^{51}Chromium Assays. The evaluation on population scale, however, lacks sensitivity and fails to identify surviving cancer cell populations. We used “in channel micropatterning” to generate arrays of homogenously distributed single cancer cells, or cancer cell aggregates (spheroids) to evaluate cytotoxic T cell activity on a single cell level. By optical analysis combined with advanced image processing of cell / spheroid arrays we obtained efficient label free analysis of each cancer cell – T cell interaction over time. Additionally, using matrix embedded arrays, highly physiological three dimensional T-cell migration conditions could be mimicked.

P1197/B324

Glutamate Receptor Signaling Improves Anti-tumor T Cell Immune Responses.

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The interaction between the nervous system and immune system has sparked interest in recent years. Emerging evidence shows an intricate neuro-immune network. We were intrigued by the expression of various neurotransmitter receptors on mouse T lymphocytes. Specifically, following TCR stimulation, glutamate receptors (GluR) were significantly upregulated on both $CD4^+$and $CD8^+$T cells, with a peak at 48 h. Concomitant with the upregulation of activation molecules CD69, CD25 and CD44, proliferating $CD8^+$ T cells presented higher levels of GluA3 and mGluR1 when compared with non-proliferating cells, as well as a higher production of IFN-γand granzyme B. By blocking group I metabotropic glutamate receptors and AMPA receptors through antagonists, we show that $CD8^+$T cells have a delayed activation but their viability is not altered. Activation markers as CD25, CD69 and CD44 were downregulated on $CD8^+$T cells but were not completely decreased. However, GluR blockade affected $CD8^+$T cells proliferation and their ability to kill tumor cells in *vivo*or target cells in *vitro*. While the frequency of $GluR^+$ $CD8^+$T cells ranges around 30% of total $CD8^+$T population, the effect of blocking glutamate signaling through receptor antagonists is major and it is mediated by transient reduction in protein phosphorylation of TCR mediated pathways as well as calcium modulation. Overall, data suggest that glutamate receptors may have a stimulatory effect on T cell activation and glutamate agonists may boost T cell response in an immunosuppressive setting such as cancer.

P1198/B325

Studying CAR T Cells in a Solid Tumor Model.

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T cells orchestrate immune responses when their receptors bind specific antigen of a pathogen or cancer. Cancer immunotherapy eliminates cancer cells primarily by enabling anti-tumor T cell activity. Chimeric antigen receptor (CAR) T cells are a revolutionary form of cellular immunotherapy. CARs are genetically engineered antigen- binding receptors that permit CAR T cells to recognize and eliminate cancer cells. While CAR T cells recognizing CD19 have proven efficacious against B cell leukemias and lymphomas, navigating the more complicated microenvironment of solid epithelial tumors is a challenge for CAR T cell therapies. Optimizing CAR T cell metabolism may permit enhanced anti-tumor function in the nutrient-deprived tumor microenvironment. To study CAR T cells in a solid tumor microenvironment, we endeavored to develop murine B16 melanoma and MC38 colorectal carcinoma cell lines that ectopically express CD19. Transduction experiments proved unsuccessful, but we will next attempt to generate CD19-expressing cell lines with a transposon system. Previous work in the Rathmell lab has demonstrated that inhibition of Glutaminase, an enzyme that converts glutamine to glutamate, alters T cell differentiation which may enhance anti-tumor immunity. We hypothesized that Glutaminase inhibition by small molecule CB-839 would alter mitochondrial metabolism, which has been associated with improved CAR T cell functions. CB-839 treatment of CD8 T cells increased mitochondrial mass without increasing mitochondrial membrane potential, suggesting enhanced mitochondrial fitness. Our results suggest that glutaminase inhibition may be a promising approach to enhance CAR T cell function in solid tumors.

P1199/B326

Macrophage Checkpoint Disruption Enhances in *Vitro* Phagocytosis and in *Vivo* Repression of Opsonized, Syngeneic Tumors in Immunocompetent Mice.

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Macrophages possess an inhibitory receptor SIRPα that binds ‘marker of self’ protein CD47 on all cells, including cancer cells, to impede their engulfment. However, C57 mice with CD47 knockout are nearly normal, and anti-CD47 is likewise ineffective in monotherapy against cancer in clinical trials whereas combination with a tumor-opsonizing monoclonal is effective in some patients. A syngeneic, immunocompetent mouse model is thus needed, and so we first deleted CD47 in B16 murine melanoma cells. B16’s are weakly immunogenic in C57 mice and are often studied for immunotherapies. Phagocytosis of knockout cells by primary mouse macrophages is enhanced more than two-fold relative to B16’s with wildtype-levels of CD47 – but only when cells are opsonized with a mouse monoclonal against melanoma antigen Tyrp1. Similar effects are seen in combinations with monoclonals against either CD47 on B16’s or SIRPα on macrophages. CD47 knockout tumors grow similar in C57 mice to wildtype tumors – which are mechanically soft and fragmentable, but growth of knockout tumors is repressed by systemic injections of anti-Tyrp1 that has no effect on wildtype tumors. In a more therapeutic approach, marrow-derived monocytes treated *ex vivo* with anti-SIRPα are co-injected with anti-Tyrp1, extending survival while avoiding anemias caused by anti-CD47 infusions. Efficacy with macrophage checkpoint blockade thus requires a simultaneous activation of macrophages.

P1200/B327

Ex Vivo Phenotyping and Potency Monitoring of CD19 Car T Cells with a Combined Flow Cytometry and Impedance-based Real Time Cell an alysis Workflow.

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Immunotherapy harnesses the immune system to attack cancer cells. While immunotherapy holds tremendous promise for cancer treatment, significant challenges remain in clinical translation. Further development is still required to expand treatment to more cancers and minimize adverse effects. T-cell differentiation and exhaustion influence the establishment and persistence of CAR-expressing T cells. Evaluation of the potency of CAR T cells is essential for effective immunotherapies. Here, we study the potency of CAR-T cells using of a combined impendence-based Real Time Cell an alysis (RTCA) and flow cytometry cell analysis workflow for *ex vivo* cytolytic potency monitoring of CD19 CAR T cells (CART19). CART19 cell killing of CD19 expressing HEK-293 cells with different E:T (Effector to Target) ratio was monitored by the xCELLigience RTCA system. Concurrently, T cell cytokine production was measured on the NovoCyte Quanteon. This workflow provides a thorough examination of CART cell function, RTCA data demonstrates that CART19 killing is highly potent as well as being antigen specific and dose dependent. In conjunction, more cytokine was produced with higher numbers of effector CART19 cells. It was also observed that CART19 cells express stem memory cell (T_{SCM}) markers, expressing activation and exhaustion markers after expansion. Co-culture with HEK-293-CD19 also results in increased expression of CD25, decreased expression of CD127 and upregulation of exhaustion markers, PD-1, TIM-3, and LAG-3. In summary, this study demonstrates the combination of RTCA and flow cytometry cell analysis is a powerful workflow for immunotherapy research and can be employed for QC in CAR T cell manufacturing.

P1201/B328

***Ganoderma Lucidum* Compounds: Effects on Inflammatory and Triple-Negative Breast Cancer Cell Viability.**

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Breast cancer is one of the leading causes of death in the United States and in Puerto Rico. Among the different types of breast cancers, the inflammatory breast cancer (IBC) and the triple-negative breast cancer (TNBC) subtypes are two of the ones with the worst prognosis, presenting with low survival rates, early recurrence and resistance to treatment. These subtypes are also seen more frequently in younger populations. TNBC is recognized by the absence of estrogen, progesterone, and human epidermal growth factor 2 receptors (ER, PR and HER2, respectively). IBC may present with different molecular subtypes, including TNBC, ER-/PR-/ HER2+, or ER+/PR+/HER2+. *Ganoderma lucidum* extract (GLE) has proven to have anti-cancer properties against IBC and TNBC, but individual compounds in GLE are yet being studied. This experiment studied one of the active compounds in GLE, ergosterol peroxide (EP) and the synthesized cholesterol peroxide (CPO), both part of the steroid family and of cholesterol core. Three cell lines, SUM-159 (TNBC), SKBR3 (HER2+ non-IBC) and SUM-190 (HER2+ IBC), were treated with increasing concentrations of EP or CPO for 72h to evaluate the capacity of these compounds to reduce cell viability in each of these lines. Our results showed a concentration dependent decrease in cell

viability in all three cell lines, with a higher anti-viability capacity for CPO (IC_{50} of 8.90µM for SUM-190, 13.84µM for SKBR3, and 30.84µM for SUM159) compared to EP (IC_{50} of 22.41µM for SUM-190, 45.14µM for SKBR3, and 31.80µM for SUM159). Also, SUM-190 IBC cells were found to be more sensitive to both compounds compared to SUM-159 and SKBR3 cell lines. These findings suggest that CPO might affect HER2 signaling, especially in IBC cells. However future studies need to be conducted in order to confirm these findings.

P1202/B329

Targeting Triple Negative Breast Cancer: a New Molecular Target and Agent.

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Triple-negative breast cancer (TNBC) is a form of breast cancer that does not express any of the three main markers for breast cancer. To better detect and treat breast cancers including TNBC, it is vital that new methods be developed. The use of targeted molecular imaging agents (TMIAs) is a promising method which can be applied to effective diagnosis of primary and metastatic tumors by locating and illuminating metastatic cell types of interest. Initial studies suggested that targeting peptides, including deca-peptide 18-4, discovered by Dr. Kaur at Chapman University, bound to keratin 1 of breast cancer (BrCa) cells. We evaluated the utility of a TMIA based on 18-4, produced by the Molecular Imaging Lab at RIT. This new TMIA was tested on MDA-MB-231 breast cancer cells and on other cancer cell lines grown in vitro and visualized with confocal fluorescence microscopy (CFM). The results show that the 18-4 based TMIA (which we have named M2) binds to breast cancer cells and is endocytosed into the cytoplasm. Developing agents that are able to effectively and accurately target and detect metastatic tumors should allow better clinical treatment and enhance cancer survival.

P1203/B330

Cytotoxic Mechanism of Palladium Complex Aj-5 in Three-dimensional Breast Cancer Culture T47-d Cell Culture.

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Metallic compounds, such as palladium exhibits significant anti-cancer activity accompanied by improved bioavailability, efficacy, and toxicity characteristics that limit the use of platinum analogues in antineoplastic chemotherapy. Despite that, new palladium complexes are being developed, but, the descriptions of cytotoxic effects in culture techniques that better mimic the tumor environment in *vivo*, such as multicellular spheroids, is still insufficient. In this context, the present study aimed to evaluate the cytotoxic response of palladium complex AJ-5 in multicellular T47-D breast tumor cell spheroids. The T47-D multicellular spheroids were cultured for 7 days by liquid overlay technique, treated for 24h with AJ-5 palladium complex at 1.6 µM, and 3.2 µM concentrations. The parameters of spheroid size, cell cycle (acquire by flow cytometry), expression of apoptosis, autophagy and adhesion proteins (caspase-3, LC3a/b, E-cadherin, respectively) by Western blot were evaluated as well as their intracellular localizations by immunofluorescence. The results showed increase in spheroids diameter treated with AJ-5, mainly at highest concentration, with mean values of 753 µm compared to control (644.9 µm), which seems to be associated with loss of cell-cell adhesion corroborated with reduction of e-cadherin protein expression. In addition, AJ-5 complex seemed to induce apoptosis and autophagy at the highest concentration by increase caspase-3 and LC3-II protein expression. In conclusion, AJ-5 palladium complex induced reduction of spheroid cell adhesion associated with increase of apoptotic and

autophagic process, corroborating previous monolayer studies. However, further studies will be needed to better characterize that cytotoxic effects in multicellular spheroids.

P1204/B331

Mechanistic Insight of Ergosterol Peroxide Against Triple-negative Breast Cancer Models.

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Triple-negative breast cancer (TNBC) accounts for approximately 10-20% of the breast cancer population and is often associated with poor patient outcome. This poor prognosis is increased due to the lack of targeted therapies available for TNBC. Former studies have identified anti-cancer effects of *Ganoderma lucidum*extract (GLE) against breast cancer models, and have attributed some of its effects to Ergosterol Peroxide (EP). This oxidized sterol possesses a therapeutic index, and has been proven to be the main cytotoxic constituent of GLE. EP has proven to affect signaling that maintains mitochondrial integrity and plays a role in cellular response to peroxides. By doing so, EP can compromise the mitochondria and induce reactive oxygen species (ROS), causing apoptosis. Although inhibitory effects of EP have been documented, mechanism of action is still unknown. Thus, the main objective of this study is to investigate EP's mechanism of action against TNBC cell models (SUM-149 and MDA-MB-231) to further develop EP as a naturally derived TNBC therapeutic. To assess our objective, the following cellular-molecular biology assays are conducted: mammalian cell culture protocols, apoptosis and cell cycle assays for CRISPR clones, migration assays, immunoprecipitation assays, and western blotting. Our results suggest that EP reduces cell migration in TNBC clones, induces apoptosis more readily, and exhibits a more potent cytotoxic effect. In conclusion, our studies will further validate EP's therapeutic potency in TNBC cell models by providing preliminary insight to its mechanism of action. This work was supported by NIH NIGMS #SC3GM111171 (MMM), and NIMHD #MD007583 (MMM).

P1205/B332

Mirna Expression Changes After Decarbomoyl Mitomycin C (dmc) Exposure in the Mcf7 Breast Cancer Cell Line.

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MicroRNAs (miRNA) are non-coding RNAs that post-transcriptionally regulate gene expression by binding functional mRNA. Altered miRNA expression has been found to be important in different tumorigenic events in breast cancer. It is currently known that mitomycins such as decarbamoylmitomycin C (DMC) are able to alkylate DNA upon reductive activation to generate monoadducts and Interstrand Crosslinks (ICLs) affecting important pathways and triggering cell death in cancer cells. However, whether cellular mitomycin effects are mediated by deregulating RNA molecules and more specifically miRNAs, has not been investigated to date. Here, we explore whether DMC has the potential to alkylate miRNA molecules and affect miRNA levels leading to changes of expression in specific mRNA targets. The identification of RNA adducts in DMC treated miRNA was performed at the nucleoside level following digestion with phosphodiesterases and phosphatases. The digest was analyzed by liquid chromatography-photo diode array detector-mass spectrometry (LC-PDA-MS). In a parallel in vitro experiment MCF7 breast cancer cells were treated with 50uM DMC for 24 hours to determine possible changes in miRNA and mRNA expression levels. The change in expression of 800

miRNAs and 770 cancer-related mRNA was determined using Nanostring RNA panels, and the miRNA Target Prediction Database (miRDB) was used to identify gene targets for each differentially expressed miRNA. We identified a DMC miRNA adduct based on its precursor mass (m/z 543.2) and UV spectra. These data were identical to those of an authentic standard synthesized independently by direct reaction between guanosine and reduced DMC. In in vitro experiments, we found that miRNAs miR-1260a, miR-125a-5p, and miR-503-5p were underexpressed after DMC exposure, and that its respective target mRNAs, THEM4, EIF4EBP1, and CCNE1 were overexpressed in the same samples. These mRNA encode for proteins known to inhibit cancer cell growth and metastasis by interfering in translation, promoting chromosome instability, or inhibiting the RET signaling pathway, respectively. Our work suggests that one potential biological mechanism of action of mitomycins is through direct interaction and deregulation of miRNA levels. Further studies will have to be conducted to confirm the presence of miRNA-DMC adducts in vivo and to determine whether their presence directly leads to changes in miRNA expression.

P1206/B333

The Flavonoids Acacetin and Pinostrobin Selectively Inhibit Cell Migration and Adhesion in Cell Models of Breast Cancer.

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Cancer cell metastasis, the process by which cancer cells migrate and form new tumors elsewhere in the body, accounts for approximately 90% of cancer-related deaths worldwide. Consequently, finding new therapies that target cancer cell metastasis is crucial for the effective treatment of late-stage cancer patients. For the last several decades, the majority of anticancer drugs have originated in some form from natural compounds. These compounds continue to be the main source of disease treatment. Naturally occurring flavonoids have been found to have anticancer effects in various cancer types. However, the effects of flavonoids have not been extensively studied on breast cancer cells. Here, we tested the effects of the flavonoids acacetin and pinostrobin on malignant MDA-MB-231 and T47D breast epithelial cells and non-tumorigenic MCF10A breast epithelial cells. Using scratch and transwell assays, both acacetin and pinostrobin selectively inhibited the migration of both MDA-MB-231 and T47D breast cancer cells in a dose-dependent manner while exhibiting blunted effects on non-tumorigenic MCF10A cells. However, neither compound had an effect on cell proliferation in any of the three cell lines when tested over a wide range of concentrations. Furthermore, both acacetin and pinostrobin reduced MDA-MB-231 and T47D cell adhesion by approximately 35-50%, but had no significant effect on MCF10A cell adhesion. Moreover, both compounds inhibited cell spreading and focal adhesion formation in the malignant but not the non-tumorigenic breast cell lines. Taken together, these results suggest that both acacetin and pinostrobin mediate their effects on motility by inhibiting adhesion in malignant breast epithelial cells. These findings position acacetin and pinostrobin to be potential drug treatments for breast cancer metastasis during late-stage tumor progression.

P1207/B334

The Covalent Cdk7 Inhibitor Enhances Temsirolimus-induced Cytotoxicity Via Autophagy Suppression in Human Renal Cell Carcinoma.

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Renal cell carcinoma (RCC) is the major cancer of the kidney. The 5-year survival rate is 74% overall and only 8% for Stage 4 diseases. In the past 10 years, several agents including tyrosine kinase inhibitors (TKIs), mTOR inhibitors and immune checkpoint inhibitors are available as first- or second-line therapy for metastatic RCC. However, the survival benefits are limited. Recently, THZ1 was identified as a CDK7 inhibitor interfering the transcriptional machinery. Although it has been shown effective in various pre-clinical models of different cancers, the data for RCC has never been reported. In our study, we performed in *vitro* and in *vivo* experiments to investigate the effect of THZ1 in RCC. We also analyzed the significance of CDK7 in our clinical cohort. Our result showed that THZ1 induced apoptosis and cell cycle arrest in RCC cells, thus reducing cell viability in *vitro*. In addition, THZ1 in combination with temsirolimus had synergistic effects in *vitro*, probably through the inhibition of autophagy. Compared with monotherapy with either THZ1 or temsirolimus, the combination treatment further suppressed tumor growth in our mice xenograft models. Finally, the impact of CDK7 expression on tumor progression and patient survival was demonstrated in our clinical cohort. These results indicated that CDK7 is associated with the progression and the prognosis of RCC, and THZ1 might potentially serve as a therapeutic agent to conquer drug resistance in RCC.

P1208/B335

Glutamine Deficiency Induces Apoptosis by Enhancing Ros and Pp2a in Lymphoma.

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Altered glutamine metabolism is emerging target of cancer therapy. Glutamine deprivation has been proposed to induce apoptosis in multiple cancers. Dependency on glutamine metabolism of skw 6.4 lymphoma cell was investigated in vitro and in vivo. Glutamine deprivation induces apoptosis of skw 6.4 cell. Intracellular concentration of metabolites in tricarboxylic acid cycle and glutathione concentration were reduced and concentration of reactive oxygen species (ROS) and expression of PP2A were increased in glutamine-deprived skw6.4 cell. Reduction of ROS by antioxidants and knockdown of PP2A inhibited apoptosis induced by glutamine deprivation. Glutamine antagonist reduces skw6.4 tumor size in mouse xenograft model in PP2A-dependent manner. Overall, these results suggest survival of skw6.4 cells depends on the supply of glutamine in PP2A-dependent manner.

P1209/B336

Increased ROS Enhanced Photosensitizer Accumulation and PDT-cytotoxicity in Highly Metastatic Breast Cancer.

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Breast cancer is one of the most common types of cancers prevalent in women. Several types of breast cancers can easily metastasize to bone and cause disease complications, such as hypercalcemia and pathologic fracture, thus compromising the quality of life of people affected by it. Bisphosphonate drugs are often used for the treatment of bone metastasis to suppress osteoclastic bone resorption. However, bisphosphonate has adverse effects on the gastrointestinal tract and kidney and also induces osteonecrosis of the jaw. Photodynamic therapy (PDT) is an alternative cancer treatment approach with minimal invasiveness. It is a combination treatment that uses photosensitizers, which accumulate in tumor cells, followed by laser irradiation. We previously reported that the cellular incorporation of protoporphyrin IX (PPIX), the 5-aminolevulinic acid (5-ALA) precursor, was regulated by reactive oxygen species derived from mitochondria (mitROS). In this study, we investigated the incorporation of 5-ALA, accumulation of PPIX, and subsequent effects on cell viability after laser irradiation of two different breast cancer cell lines with different metastaticity. The highly metastatic breast cancer cell line 4T1E/M3 showed a significant increase in ROS production after treatment with indomethacin (IND). In addition, IND treatment enhanced the cellular uptake of 5-ALA via PEPT1 upregulation in 4T1E/M3, but not in the non-metastatic cell line. Overall, metastatic breast cancer is likely to be sensitive to ROS and activate signaling pathways associated with 5-ALA transportation, suggesting that ALA-PDT could be an effective treatment for metastatic breast cancer with low invasiveness.

P1210/B337

Crotamine Reduces Migration and Invasion of Breast Cancer Cells Via the Mmp-9 Activation.

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Breast cancer is leading cause of death by cancer in women in world. Development of therapeutic modalities targeting the key molecules in cancer progression is crucial to improve the survival rates of breast cancer patients. Crotamine, a peptide toxin isolated from snake venom, has cytotoxicity effects on cells and anti-microbial activities. However, effects of crotamine on cancer has not been extensively studied. Crotamine treatment showed the no significant cytotoxicity on highly metastatic breast cancer cell line, MDA-MB231 at 30 μM and lower concentrations. At 10 μM and 20 μM, the crotamine significantly reduced the migration and invasion of EGF-stimulated MDA-MB231. Crotamine treatment reduces the activity and expression of MMP-9 and expression of active forms of AKT, ERK, c-JUN, and p65 in EGF-stimulated cells. Reporter assays showed crotamine attenuated the induction of NF-κB and AP-1-depenedent promoter activities and proximal MMP-9 promoter activity. Crotamine repressed the invasion, migration, and activation of signal pathways in primary triple negative breast cancer cells in the same way of MDA-MB231. Overall, these results suggest the anti-breast cancer effects of crotamine and

provide the support for the further application of crotamine and its derivatives in anti-cancer treatments.

P1211/B338

Parthenolide Exerts a Cytotoxic Effect in Triple Negative Breast Cancer Cells through Interrupting Interferon-alpha Signaling.

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Triple negative breast tumors (TNBC) lack the estrogen receptor, progesterone receptor and the human epidermal growth factor receptor and are responsible for approximately 10-25% total breast cancer diagnoses. Heterogeneity is inherent in TNBC as delineated by the identification of multiple subtypes based on gene expression analysis including basal, mesenchymal, immune-enriched and luminal androgen receptor (AR) subtype. The tumoral diversity and lack of molecular targets hinder the development of FDA approved specific targets, perhaps resulting in the lower 5 year overall survival rate compared to receptor positive breast tumors due to metastatic recurrence. Therefore, identification of novel targets and therapeutic agents is necessary to further enhance therapy as well as to understand TNBC biology. Our investigations identified that an interferon-alpha (IFN-alpha) regulated gene, interferon inducible transmembrane protein-1 (IFITM1), is overexpressed in TNBC cell lines. IFITM1 regulates the aggressive phenotype of TNBC cells in *vitro and* in *vivo*. To date, the only known drug to target IFITM1 is the JAK inhibitor Ruxolitinib, which has had poor clinical outcomes in patients with TNBC. Therefore, we used a high-throughput FDA approved drug screen to identify agents which are both toxic to TNBC cells as well as target IFITM1. Parthenolide (PN), a NF-kappaB inhibitor, was identified as a drug of particular interest. In this study, we investigated the functional role of targeting IFITM1 and subsequent PN sensitivity on multiple TNBC cell lines. We aimed to define the mechanism of PN and its effects in TNBC cell lines that have yet to be studied. Our data suggest that both the basal-like subtype of TNBC (SUM149 and MDA-MB-468) and the mesenchymal subtype of TNBC (MDA-MB-157) are sensitive to PN. Phenotypically, it was identified that both inhibition of IFITM1 and treatment with the IC50 of PN significantly decreases migration, growth and clonogenicity in all three TNBC cell lines. Notably, we identified that PN regulates IFITM1 expression on both the mRNA and protein level. Lastly, we identified a novel mechanism of PN such that it inhibits IFN-alpha signaling through inhibiting IFN-alpha gene expression and by interrupting its downstream signaling and subsequent activation of IFITM1, thus resulting in increased apoptosis. These results suggest that the IFN-alpha signaling pathway and subsequent IFITM1 expression has pro-survival mechanisms, that there is intracellular crosstalk regulated by IFN-alpha in TNBC, and that PN is a potential therapeutic agent for TNBC regardless of clinical subtype.

P1212/B339

Inhibition of Mcf-7 Breast Cancer Cell Proliferation and Cell Cycle Progression by the Dietary Polyphenols Ellagic Acid and Luteolin.

J. Rivosecchi-Fulton, **K. Baker**; University of Indianapolis, Indianapolis, IN.

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Epidemiological studies have demonstrated a positive correlation between the consumption of polyphenolic compounds and cancer prevention. Ellagic acid and luteolin, dietary polyphenols found in a variety of fruits and vegetables, have been shown to inhibit cancer cell proliferation and motility and induce apoptosis in *vitro*. In this study, we evaluated the antiproliferative effects of ellagic acid and luteolin, individually and in combination, using MCF-7 breast cancer cells. We found that both ellagic acid and luteolin inhibited MCF-7 cell proliferation in a dose dependent manner. Furthermore, we found that co-administration of ellagic acid and luteolin led to a greater inhibition of cell proliferation than either agent alone at the same dose and indicate an additive effect. These results suggest that combinatorial treatments using ellagic acid and luteolin may be an effective chemotherapeutic strategy against breast cancer.

P1213/B340

Agents That Decluster Centrosomes Are Effective Against an euploid B-Cell Precursor ALL Cells.

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Background: Acute lymphoblastic leukemia (ALL) is the most common malignancy in children. Some ALL subtypes are defined by aneuploidy, which is likely caused by a defective cell division that also bestows an abnormal number or size of centrosomes. Cancer cells, but not normal cells, must cluster these organelles during cell division. Thus, centrosome clustering (CC) inhibitors may specifically kill B-ALL cells. Tumor cells generally respond to targeted therapies but often a population of cells becomes refractory. We hypothesized that pediatric ALL may frequently contain centrosome amplification and be sensitive to CC inhibitors; as CC inhibitors are likely to induce genome instability, B-ALL cells refractory to CC inhibitors may activate innate immunity via cyclic GMP-AMP synthase - stimulator of interferon genes (cGAS-STING) pathway. **Methods**: Twelve potential CC inhibitors were screened against a panel of four immortal B-ALL cell lines. Four CC inhibitors were selected for further study based upon correlation between IC50 dose and frequency of centrosome amplification. Primary B-ALL cells, both patient samples and cells isolated from a transgenic Emu-ret mouse that spontaneously develops leukemia, were grown as co-culture with feeders and screened for centrosome amplification and responses to CC inhibitors; primary human normal stem cells were also counter-screened. Immortal 289 mouse cells (Emu-ret+ B-ALL) that resist CC inhibitors were derived over a period of four months. Centrosome phenotype, spindle morphology, cGAS colocalization, gamma-H2AX and micronuclei were determined using immunofluorescence. Colorimetric assay and high content screening were performed to measure drug efficacy. **Results**: Centrosome amplification (CA) is a common feature of human diagnostic and relapsed B-ALL samples. CC inhibitors induced multipolar spindles, and CA levels correlated with sensitivity to CC inhibitors in primary patient B-ALL samples and normal stem cell control samples. The refractory B-ALL cells (termed 289r) showed increased level of gamma-H2AX and micronuclei. cGAS was found to colocalize to micronuclei. Primary B-ALL cells also showed increased levels of cGAS- MN after treatment with CC inhibitors. **Conclusion**: IC50 doses for CC inhibitors are 10 - 100 X lower for primary human pediatric B-ALL than normal control stem cell samples. Immortal refractory B-ALL cells have increased levels of genetic instability, as measured by gamma-H2AX, and genomic instability, as measured by micronuclei, which elevates the level of cGAS-bound micronuclei and may activate

TBK1/NF-$_K$B and pro-inflammatory signals. The combination of CC inhibitors, such as PARP inhibitors, and immune checkpoint inhibitors may be effective against relapsed pediatric B-ALL samples.

P1214/B341

Developing a Single-Cell, Imaging Assay to Characterize CAR-T Functional Heterogeneity.

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Chimeric antigen receptor (CAR) T cells are genetically modified T cells designed for antigen specific killing of cancer cells. CAR-T populations are typically composed of both transduced and un-transduced cells. Heterogeneity in the donor cells and transduction process contribute to variability to CAR-T function and viability. There is a need to understand the subpopulation of cells which comprise a CAR-T population. This information could provide insight into the effectiveness of a CAR-T therapy. We developed a large field of view, live cell imaging workflow using phase contrast microscopy, fluorescence surface marker staining, and machine learning to perform single cell characterization of CAR-T cells by imaging in microgrids. This approach is similar to the TIMING assay (Vadarajan lab, University of Houston) to identify dynamic cytotoxicity characteristics determined from live cell imaging. Using a model CAR-T cell system targeting human epidermal growth factor receptor 2 (HER2), we were able to identify subpopulations in CAR-T and un-transduced T cells based on differences in viability and proliferation rates. Single cell analysis of CAR-T cells revealed subpopulations where 8.5% were apoptotic and 65% were proliferative with a doubling time of 52.0 ± 34.9 hours (mean ± standard deviation). Single cell analysis of un-transduced T cells showed subpopulations where 17% were apoptotic and 36% were proliferative with a doubling time of 56.4 ± 32.7 hours. Future work includes staining surface markers to differentiate the phenotype of the apoptotic and proliferative CAR-T subpopulations. Moreover, quantification of cytotoxic CAR-T heterogeneity is being evaluated by co-culturing CAR-T with target cells in microgrids and quantifying single cell interactions. This approach can be applied to other CAR-T populations to understand what subpopulations exist in a heterogeneous cell population and potentially enable enrichment of subpopulations with desirable dynamic characteristics determined from live cell imaging.

P1215/B342

Appetizers & Entrees: Phagocytosis of Microbeads Or Red Blood Cells (RBCs) and Then 'Self'-Inhibited Cancer Cells.

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CD47 is a ubiquitous membrane protein that functions as a marker of 'self' to enable immune recognition of healthy cells by SIRPα on macrophages. Combining blockade of this inhibitory interaction with pro-phagocytic cues on cancer cells is already emerging in clinical therapy, but many challenges remain including control over the amount of eating. Here, depletion of CD47 via CRISPR knockout (KO) in B16 melanoma cells is combined with B16 opsonization by anti-Tyrp1, which results in more eating of cancer cells in *vitro* by mouse bone marrow-derived macrophages (BMDMs) relative to wild-type (WT) and relative to un-opsonized B16s. Pre-feeding BMDMs with ~2 μm polystyrene microbeads 24 hours prior to feeding KO's leads to slightly greater eating of the cancer cells, whereas pre-feeding of RBCs eliminates such phagocytosis. In *vivo*, intravenous injection of these same KO cells into C57 mice results

in lung tumor nodules that model metastasis and that are indistinguishable from WT tumors. However, we find anti-Tyrp1 injections suppress the growth of CD47 KO tumors but not WT tumors - which reinforces the need for combining pro-phagocytic signals and blockade of CD47. For a more therapeutically relevant approach, we make antibody Primed & Phagocytosis Blocked macrophages (A'PBs) by antibody-treatment of donor marrow monocytes to block SIRPα and also to target B16s via anti-Tyrp1. WT lung tumor nodules are significantly reduced in size and number by A'PBs, and studies of A'PB that have pre-eaten microbeads or RBCs before being infused are now testing the hypothesis that such 'appetizers' affect the trafficking and cancer-engulfment capacity of these immune cells.

P1216/B343

Enhancing Cytotoxicity of Natural Killer Cells by Exogenous Mitochondrial Transfer.

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Natural killer (NK) cells have been concentrated as a source of anti-cancer cell therapies as a major component of the innate immune system, contributing to the first line of defense against cancer and viral infections. Mitochondria are organelles responsible for cellular function, including energy metabolism, free radical production, cell survival and death. Recently, we have developed a simple and easy mitochondrial delivery protocol using centrifugation, in which mitochondria can be delivered directly to target cells. Here, we delivered mitochondria directly to NK cells to enhance NK cell cytotoxicity. Mitochondria-enriched NK cells have shown a potent therapeutic effect in myeloid leukemia models in *vitro*. As a result, our findings showed a new strategy for NK activation methods that could be used clinically.

P1217/B344

Characterization of Coumestrol as an Anticancer Therapy Against Triple-Negative Inflammatory Breast Cancer.

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Breast cancer is the second leading cause of death in females in the United States, being Inflammatory Breast Cancer (IBC) one of the most aggressive subtypes. The poor prognosis for patients with IBC emphasizes the need to better understand the molecular signature. To this date, there is no effective targeted therapeutics, especially for those patients that account for approximately 20-40% of IBC cases with triple-negative breast cancer (TNBC) classification. Importantly, several studies have shown that estrogen can exert non-genomic effects in IBC and non-IBC TNBC, mediated by the expression of a shorter isoform of the canonical estrogen receptor alpha (ER), named estrogen receptor alpha-36 (ERα-36), and a G protein-coupled receptor, GPR30, that can also be activated by estrogens. In this context, estrogen can activate rapid non-genomic signaling pathways involved in the acquisition of oncogenic phenotypes such as increased motility and invasion in IBC cells. Phytoestrogens, like coumestrol (Cou), are natural compounds found in plants, specifically soybeans. Also, it is a polyphenolic compound that is structurally similar to estradiol (E2), and several studies have shown that coumestrol inhibits cell proliferation and migration in TNBCs. We hypothesize that (1) estrogen non-genomic signaling has an active role in the aggressive metastatic phenotype of IBC, and (2) Cou has anticancer activity by inhibiting estrogen non-genomic signaling in IBC. To test these hypotheses, IBC cells, SUM 149, were

treated with E2 and activation of downstream kinases (ERK and AKT) was analyzed by Western blot. Immunofluorescence in SUM149 cells and Western Blot showed expression of both, ERα-36 and GPR30. Besides, preliminary observations treatment using previously reported IC50 (50μM) of Cou caused a decrease in cell viability in ER-positive breast cancer cell lines and TNBC, including IBC cells. Also, in comparison to E2, Cou decrease migration in IBC cells. In summary, IBC cells are responsive to E2 treatment making this signaling pathway attractive for the development of innovative therapeutic strategies. Importantly, preliminary studies show a potential anticancer activity of coumestrol in IBC cell lines by either decreasing migration and/or cell viability.

P1218/B345

Protect Or Potentiate: the Role of Common Medications in Modulating Response to Radiation Exposure.

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Objective: to investigate the effects of common medications on protein signal transduction and metabolic activity in response to radiation in a lung cancer cell line model. Drugs and drug metabolites are known to modulate cellular signaling cascades, either intentionally or unintentionally as off-target effects. For example, calcium channel blockers and angiotensin converting enzyme inhibitors are known to confer cellular protection from radiation. Radiation also modulates cell signaling pathways in both target cells and distant cells (by-stander effect). We examined cell viability and protein signaling pathways following escalating radiation doses, with or without drug treatment. Methods: NCI-H2228 non-small cell lung cancer cells were x-ray irradiated at various doses with and without *a priori* drug treatment. We evaluated medications used for the treatment of cardiovascular ailments, high cholesterol, epilepsy, and diabetes. Treatment consisted of physiologic doses of a calcium channel blocker, an angiotensin converting enzyme (ACE) inhibitor, a HMG CoA reductase inhibitor, a beta-adrenergic antagonist, an anti-epileptic drug, and 1,1-dimethylbiguanide hydrochloride. Cell viability/metabolic activity was measured by quantifying the reduction of 10% resazurin in cell culture. Signal transduction pathways were quantified by reverse phase protein array. Immunohistochemistry of cytospin preps was used to assess apoptosis. Results: Untreated cells were irradiated with doses ranging from 0.1 Gy to 20 Gy. Effects became pronounced by Day 6. Maximal effects were observed with 20 Gy of radiation, with half maximal effects observed at 2.5 Gy. A time course of 2.5 Gy post exposure toxicity shows persistent decline of viability over 2 weeks. Leviteracetam and metformin alone increased sensitivity to low dose radiation. We identified propranolol and an ACE inhibitor + metformin regimen as agents that provide temporary resistance against low-dose (2.5 Gy) radiation. Cleaved PARP was present in Metformin+ACE treated cells 20 days post-irradiation. Conclusion: Medications used for the treatment of cardiovascular ailments, high cholesterol, epilepsy, and diabetes can protect lung cancer cells, or potentiate the effects, of low dose radiation. The effects of common medications were reduced when high doses of radiation were applied.

Chromatin and Chromosome Organization 1

P1219/B347

Creating an Integrated Landscape of Human iPSC Nuclear States.

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The Allen Institute for Cell Science is developing a state space of stem cell structural signatures to understand the principles by which cells reorganize as they traverse the cell cycle and differentiate. To do this, we have developed a pipeline that generates high-replicate, dynamic image data of cell organization and activities in human induced pluripotent stem cell (hiPSC) lines (the Allen Cell Collection at www.allencell.org). Each of the ~35 lines expresses an endogenous monoallelic EGFP-tagged protein that represents a particular cellular organelle or structure. For each line, we develop image-based assays and segmentation algorithms for quantitative analyses, taking advantage of the thousands of replicate high resolution 3D images for each structure. This image database is used for data and machine learning analyses and computational modeling. We are applying these same approaches to creating an integrated state space of nuclear signatures. We have endogenously EGFP-tagged an initial set of 17 nuclear proteins representing key nuclear organizational landmarks at multiple spatial scales including at the level of the nucleus and nucleoplasm as a whole (nuclear lamina, nuclear pores, several nucleolar subcompartments, and nuclear speckles), chromatin structure (histone H2B, HP1-beta, and EZH2), key proteins in chromatin looping (CTCF, SMC1A), two types of chromatin loci (telomeres and DNA replication sites), RNA polymerase, and two pluripotency transcription factors. We have imaged the sub-nuclear localization of these structures as the cells progress through the cell cycle and differentiate into cardiomyocytes. We are developing assays to image and quantify the protein localization patterns of these proteins at different spatial scales and creating machine learning transfer functions to enhance the resolution capabilities for live-cell imaging of nuclear structures. We are also developing methods to track and quantify specific loci within the genome relative to these protein landmarks. The cell lines, image data and analyses are being integrated with genome-wide assays of chromatin architecture together with the 4D Nucleome Project NOFIC centers, and once fully characterized, will be distributed publicly. For example, to provide realistic spatial constraints within the nucleus for more accurate modeling of chromatin organization basd on genomic assays, we are applying deep-learning based “label-free” technology to generate integrated models of key nuclear landmarks, beginning with the nuclear envelope, nucleolus, and nuclear speckles.

P1220/B348

The Biological Role of an Endogenous Epigenetic Metabolic Small Molecule, O-acetyl-ADP-ribose, on Yeast Silent Heterochromatin.

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The biological role of an endogenous epigenetic metabolic small molecule, O-acetyl-ADP-ribose, on yeast silent heterochromatin Shu-Yun Tung[1,#], Sue-Hong Wang[2,#], Sue-Ping Lee[1,#], Ming-Shiun Tsai[3], Gunn-Guang Liou[1,4,5,*] [1] Institute of Molecular Biology, Academia Sinica, Taipei 115, Taiwan, ROC. [2]Department of Biomedical Sciences, Chung Shan Medical University, & Department of Medical Research, Chung Shan Medical University Hospital, Taichung 402, Taiwan, ROC. [3]Department of Food Science and Biotechnology, Da-Yeh University, Changhua 515, Taiwan, ROC. [4]Institute of Biological

Chemistry, Academia Sinica, Taipei 115, Taiwan, ROC. [5]Guang EM Laboratory, New Taipei 242, Taiwan, ROC. [#]These authors contributed equally to this work Budding yeast heterochromatin is a major model to study the epigenetic gene silencing. The formation of yeast heterochromatin combines an NAD-dependent histone deacetylation and the generation of an epigenetic metabolic small molecule, *O*-acetyl-ADP-ribose (AAR) by Sir2 with SIR complexes conformational change, and follows by the repeated recruitment cycle of SIR complexes along the adjoin chromatin, and additional processing of maturation and maintenance. In our study, we show that AAR is able to enhance the silencing effect and able to promote the Sir3 spreading alone the telomeres. We also demonstrate that AAR physical associates with the Sir3 and importantly, we find that AAR is able to stabilize the association of Sir3 BAH domain with Sir3 C-terminal region. This AAR-dependent association of artificially separated Sir3 BAH domain with the rest C-terminal region that might be the key answer why the structure rearrangement of SIR complex happens when Sir2 deacetylates histone, generates AAR and interacts with Sir3. Furthermore, we also demonstrate that PolySir3-AAR plays a specific and essential role in modulating the formation of SIR-nucleosome pre-heterochromatin filaments *in vitro*. Our data suggests that for the assembly of SIR-nucleosome pre-heterochromatin filament, the structural rearrangement of SIR complexes is important and result in creating more stable interactions of Sir3, such as the inter-molecule Sir3-Sir3 interaction, and the Sir3-nucleosome interaction within the filaments. In summary, AAR, an endogenous metabolic small molecule, mediates a conformational change of protein complex that is involved in the epigenetic gene silencing.

P1221/B349

Toward a Mechanistic Characterization of Promoter Progression for Glucocorticoid Receptor Responsive Genes.

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After hormone activation, the Glucocorticoid Receptor (GR), translocates to the nucleus and binds to GR Responsive Elements (GREs) to regulate target genes. Although GR can either up or down-regulate transcripts, it has also been demonstrated that responsive genes are subjected to complex regulation profiles early after hormone treatment, with a gene specific alternance of activation and repression phases. To understand the mechanisms behind these different expression profiles, we performed a quantitative proteomic study of GR partners present on the chromatin (Chip-SICAP) at varying times after GR induction. These experiments provide a list of interactants differentially recruited to GR at the chromatin level. We alsodeveloped an RNA-Hybridization Chain Reaction (RNA-HCR) fish protocol to examine the different expression profiles of various GR responsive genes (Mt2, Ccl2, Tsc22d3) on a High-Throughput platform (HiTIF). iRNA screens against the differentially GR recruited partners will identify key players in the GR regulated expressions patterns. These approaches will form the basis for a comprehensive understanding of regulatory mechanisms active in these complex induction/repression schemes.

P1222/B350

Cohesin Impedes Heterochromatin Assembly in Fission Yeast Cells Lacking Pds5.

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The fission yeast *Schizosaccharomyces pombe* is a powerful genetic model system for uncovering fundamental principles of heterochromatin assembly and epigenetic inheritance of chromatin states. Heterochromatin defined by histone H3 lysine 9 methylation and HP1 proteins coats large chromosomal domains at the centromeres, telomeres and the mating type (*mat*) locus. Although genetic and biochemical studies have provided valuable insights into heterochromatin assembly, many key mechanistic details remain unclear. Here we use a sensitized reporter system at the *mat*locus to screen the gene deletion library to identify additional factors affecting heterochromatic silencing. In addition to known components of heterochromatin assembly pathways, our screen identified eight new factors including the cohesin-associated protein Pds5. We find that Pds5 enriched throughout heterochromatin domains is required for proper maintenance of heterochromatin. This function of Pds5 requires its associated Eso1 acetyltransferase implicated in acetylation of cohesin. Indeed, introducing an acetylation-mimicking mutation in a cohesin subunit can suppress defects in heterochromatin assembly in *pds5Δ and eso1Δ* cells. Our results show that in cells lacking Pds5, cohesin interferes with heterochromatin assembly. Supporting this, eliminating cohesin from the *mat* locus in *pds5Δ* mutant restores both heterochromatin assembly and gene silencing. These analyses highlight an unexpected requirement for Pds5 in ensuring proper coordination between cohesin and heterochromatin factors to effectively maintain gene silencing.

P1223/B351

Sumoylation-induced Liquid Condensation Clusters Telomeres.

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Telomerase-free cancer cells rely on an alternative lengthening of telomeres (ALT) pathway that employs homologous recombination (HR) to maintain telomere length for immortality. One of the unique features of ALT is the sumoylation of proteins on telomeres, which is required for telomere maintenance but the underlying mechanism remains unknown. We hypothesize that sumoylation induces liquid condensation at telomeres for ALT, based on the observations that sumoylated proteins interact with proteins containing SIM (SUMO interaction motif) and multivalent interactions between SUMO and SIM drive liquid-liquid phase separation in vitro. The induced SUMO-SIM condensates can potentially coalesce to cluster telomeres together so one can use another as a template for recombination. To test this hypothesis, we recruited SUMO ligase to telomeres with chemical dimerizers, which induced sumoylation and liquid condensation at telomeres and led to telomere clustering. To confirm that the underlying driving force is SUMO-SIM interaction, we bypassed the sumoylation process and recruited SUMO or SIM directly to telomeres and drove condensation and telomere clustering. Our work shows that sumoylation contributes to HR-based ALT by inducing liquid condensation to aid homology search, suggesting a similar mechanism may at play during normal HR.

P1224/B352

Investigating the Role of Dsk1 and Kic1 Protein Kinases in Heterochromatin Formation.

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Dis1- suppressing protein kinase (Dsk1) in *Schizosaccharomyces pombe* is a highly evolutionarily conserved serine-threonine protein kinase, implicated in important cellular functions such as mitotic cell cycle regulation, cell proliferation and differentiation. While the significance of Dsk1 on these cellular events has been well documented, the targets through which Dsk1 mediates these events is still not well understood. To investigate the potential targets of Dsk1, we used a proteomics based approach to determine the differential protein phosphorylation between wild type and dsk1-deletion mutant strains. This resulted in a list of potential Dsk1 phosphoprotein substrates that were found to be significantly associated with 10 Gene Ontology processes such as cytoskeletal and mitotic spindle organization, chromosome localization and cell cycle regulation. Physical interaction networks generated for proteins involved in these GO processes, suggest potential central nodes through which Dsk1 may affect such processes as cell growth polarity, mitotic phase transition, sister-chromatid segregation and cytokinesis. We then performed genome-wide epistasis analysis, which helped us identify a strong genetic interaction between Dsk1 and components of an integral kinetochore associated DASH complex. These results suggest that Dsk1 is likely involved either functionally or organizationally with centromeric heterochromatin. We further investigated the effect of Dsk1 as well as another LAMMER-related kinase Kic1, on centromeric gene expression in *S. pombe*. The results imply that both Dsk1 and Kic1 kinases are required for gene silencing at centromeres. Additionally, we looked at the change in phosphorylation status of a heterochromatin binding protein, Swi6 (*S. pombe* homolog of human HP1) in Dsk1 and Kic1 deletion backgrounds. The results indicate that both Dsk1 and Kic1 kinases, either directly or indirectly through a phosphorylation cascade, are involved in regulating Swi6 activity to ultimately affect heterochromatin formation/function. We also performed immunofluorescence analysis on WT cells, and observed a strong co-localization of GFP-Kic1 with Swi6 associated heterochromatic regions within the *S. pombe* nucleus. Collectively, our results provide evidence for Dsk1 and Kic1 kinases affecting chromatin structure and gene expression at the centromeric heterochromatin regions in S. *pombe* cells.

P1225/B353

Investigation of the Mechanism of Set1-mediated Telomere Silencing and Maintenance in *Saccharomyces Cerevisiae*.

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Telomeres, the nucleoprotein structures found at the ends of linear chromosomes, provide a protective function for the genome. Alteration to the chromatin structure at or adjacent to these protective caps leads to dysfunctional telomeres, which are associated with genomic instability and implicated in cellular aging and many types of cancer. In *Saccharomyces cerevisiae*, the H3K4 methyltransferase Set1 has been linked to both transcriptional activation at gene promoters and silencing at telomeres. However, while we have some insight regarding the role for Set1 in silencing, the mechanism is still largely unclear. Previously, we investigated the role of Set1 in the regulation of gene repression at native telomeres in Saccharomyces cerevisiae and found that gene derepression in the absence of Set1 is largely through a Sir protein-independent mechanism. Additionally, we have analyzed the transcriptomes from cells lacking Set1 and have found them to show a strong correlation with transcriptomes belonging to mutants that display defects in telomere maintenance, revealing a specific

role for Set1 in pathways required for telomere integrity, rather than just gene silencing. In order to further dissect the role of Set1 at telomeres, we have generated a number of Set1 mutants to determine the contributions of both its catalytic and non-catalytic activity and have characterized the role of Set1's catalytic and non-catalytic functions in telomere silencing, telomere length, and the response to DNA damage. We have determined that these phenotypes are largely dependent on Set1's methyltransferase activity. We also have preliminary data suggesting that the expression and localization of some of the protein machinery required to protect telomeres, including the telomere -binding proteins Rap1 and Cdc13, may be altered in the absence of Set1. Overall, our data provides support for a role for Set1 and H3K4 methylation in the promotion of telomere integrity through the regulation of telomere-binding proteins.

P1226/B354

Phase-separated Heterochromatin Domains Impart Mechanical Stiffness to the Nucleus.

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The association of heterochromatin with the nuclear periphery has long been suggested to reflect its transcriptional repression. However, our prior work revealed that chromatin, particularly regions of the genome associated with the inner nuclear membrane, also play an integral role in supporting the mechanical robustness of the nucleus through mechanisms that remain poorly understood. In light of new insights into the potential for heterochromatin to form a distinct liquid-like phase, we sought to define the underlying basis for the biophysical contribution of heterochromatin to nuclear mechanics. Our approach employs an image reconstruction software we developed to quantitatively measure dynamic nuclear envelope fluctuations, a novel force spectroscopy assay that employs optical tweezers to directly measure the viscoelastic properties of isolated nuclei, and microscopy approaches to monitor the dynamics of heterochromatin foci in living cells. We find that loss of the histone H3K9me2/3 binding protein Swi6, an HP1 orthologue found to undergo liquid-liquid phase separation in *vitro*, results in increased nuclear deformability in *vivo* and a 50 percent drop in the nuclear spring constant in *vitro*. Accordingly, deletion of the histone H3K9 demethylase Epe1, which drives heterochromatin spreading, results in stiffer nuclei. As cytoplasmic microtubules are coupled to the centromeres in fission yeast, we characterized the deformation of the centromeric heterochromatin in response to force, which reinforces the liquid-like behavior of heterochromatic foci. This behavior is altered in cells harboring an allele of Swi6 with a mutation in the critical "hinge" domain; this mutated protein associates normally with H3K9me2/3-rich chromatin domains but fails to induce silencing, suggesting that this allele may uncouple the phase separation behavior of Swi6 from its other biochemical activities. Accordingly, both this allele and cells lacking Swi6 demonstrate defects in the coalescence of heterochromatic foci. Taken together, these results suggest that liquid-liquid phase separation can contribute to cellular mechanics, in this case by contributing to nuclear stiffness.

P1227/B355

Phase Separation of YAP Reorganizes Genome Topology for Long-term Yap Target Gene Expression.

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Yes-associated Protein (YAP) is a transcriptional co-activator that regulates cell proliferation and survival by binding to a selective set of enhancers for potent target gene activation, but how YAP coordinates these transcriptional responses is unknown. Here, we demonstrate that YAP forms liquid-like condensates in the nucleus in response to macromolecular crowding. Formed within seconds of hyperosmotic stress, YAP condensates compartmentalized YAP's transcription factor TEAD1 along with other YAP-related transcription co-activators, including TAZ, and subsequently induced transcription of YAP-specific proliferation genes. Super-resolution imaging using Assay for Transposase Accessible Chromatin with photoactivated localization microscopy (ATAC-PALM) revealed that YAP nuclear condensates were areas enriched in accessible chromatin domains organized as super-enhancers. Initially devoid of RNA Polymerase II (Pol II), the accessible chromatin domains later acquired RNA Pol II, producing newly transcribed RNA. Removal of YAP's intrinsically-disordered transcription activation domain (TAD) prevented YAP condensate formation and diminished downstream YAP signaling. Thus, dynamic changes in genome organization and gene activation during YAP reprogramming is mediated by liquid-liquid phase separation.

P1228/B356

Impact of 3D-Genome Organization on Regenerative Ability.

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Although the field of regenerative medicine has made several advances, the molecular mechanisms driving the remarkable processes of wound healing and tissue replacement are yet to be fully uncovered. We aim to determine why new neurons do not form after spinal cord injury in higher vertebrates, by taking a closer look at the spatial organization of DNA in neural stem cells (NSCs), which differentiate into neurons during early development. DNA is arranged non-randomly within a cell nucleus, and its intricate packaging into topological domains plays a critical role in a critical role in gene regulation and thus, cell fate determination. The African-clawed frog, *Xenopus laevis*, which has high regenerative potential during its larval or tadpole stages, but loses it after metamorphosis, will serve as an excellent model organism to understand how the three-dimensional (3D) genome organization of NSCs varies in regenerative versus non-regenerative environments. We hypothesize that the 3D genome organization of NSCs alters unfavorably as *X. laevis* transitions from larval to adult stages, limiting NSC proliferative capacity and resulting in loss of neuron regeneration. To test this hypothesis, a Sox2-GFP transgenic *X. laevis* line will be created to fluorescently label NSCs. Spinal cord transections will be performed on tadpoles (staged 50) and post-metamorphosis froglets (staged 66), as representatives of regenerative and non-regenerative phases respectively. NSCs will be isolated by FACS (fluorescence activated cell sorting), and their 3D genome organization will be analyzed by the chromosome conformation technique, Hi-C. If the Hi-C data confirms our hypothesis, we will modulate specific architectural proteins, such as CTCF and cohesin, in adult *X. laevis* NSCs to test whether these modifications can restore regenerative capacity in the metamorphosed frog.

P1229/B357

Distinct Roles of the H2A.Z Variants in Chromatin Organisation and Mitosis.

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Gene silencing through heterochromatin is an important mechanism to ensure establishment of cell type-specific gene expression patterns and dysregulation of heterochromatin can result in severe developmental defects. While H3K9me3 is important for HP1 recruitment to heterochromatin, it is becoming apparent that it is not solely sufficient, thus suggesting that additional factors also contribute to direct the chromatin-binding activity and function of HP1. Some reports suggest that specific histone variants could be involved in promoting HP1 association to chromatin. One candidate is the histone variant H2A.Z. Using a proteogenomic approach to identify histone variants specifically enriched in the chromatin fraction able to bind HP1α, we have identified the H3.3 and the H2A.Z variants. In vertebrates, H2A.Z exists in two different isoforms H2A.Z-1 and H2A.Z-2 coded by two non-allelic genes, H2AFZ and H2AFV, respectively. These seem to have no redundant functions and it is not known which one is important for the establishment of heterochromatin. Moreover, compelling in *vivo* data that supports a mechanism linking these variants to H3K9me3, HP1 and heterochromatin establishment is still elusive. The analyses of DNA sequencing recovered from the proteogenomic study revealed that 50% of the HP1α bound chromatin is also bound by H3K9me3 and that 25% of the HP1α bound regions are bound by H2A.Z but only 17% of those regions contain both H2A.Z and H3K9me3. This suggests that these regions may represent different chromatin states to which HP1α can potentially bind. Moreover, we could successfully deplete each isoform in HeLa cells and demonstrate that only the H2A.Z-2 variant has an effect on HP1α localization, while H2A.Z-1 presented a strong phenotype affecting nuclear shape. Finally, the RNA seq analyses of cells depleted of each isoform and the associated mitotic phenotypes indicate a distinct and non-redundant role for the two H2A.Z isoforms in chromatin organization in human cells.

P1230/B358

A New Hi-C Method Reveals the Conformation of Sister Chromatids.

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The three-dimensional organization of chromosomal DNA is key for proper gene regulation, DNA recombination and repair, and chromosome segregation. Throughout interphase, cohesin forms DNA loops to shape topologically associated domains (TADs). Moreover, a subset of cohesin complexes links replicated sister chromatids from S-phase until mitosis to enable DNA damage repair and faithful chromosome segregation. The genomic landscape of chromatin loops and TADs has been studied in great detail by chromosome conformation capture techniques (Hi-C), but available methods cannot distinguish between identical DNA copies of replicated chromosomes. The distribution of sister chromatid linkages has hence remained unclear. We have developed a sister-chromatid-sensitive Hi-C method based on in vivo DNA labeling with nucleotide analogues. Using this methodology, we have generated genome-wide conformation maps of replicated chromosomes from interphase and mitotic

cells. We discovered four-way junctions where loops from both sister chromatids are linked at their base, as well as unlinked loop structures on individual sister chromatids. We have also applied our methodology to study reorganization of sister chromatids during cell cycle progression and will discuss the implications for chromosome segregation.

P1231/B359

Reconstitution of Cohesin and Condensin-mediated Dna Loop Extrusion in Xenopus Egg Extracts.

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The structural maintenance of chromosomes (SMC) complexes, cohesin and condensin, organize chromatin in metaphase and interphase. Condensin can extrude DNA loops *in vitro*, which may be responsible for folding chromatin into condensed chromatids during metaphase. Cohesin has been hypothesized to organize interphase chromatin into topological associating domains also via a loop extrusion mechanism. However, direct imaging of loop extrusion in cellular contexts is lacking, and consequently whether loop extrusion is a general mechanism that organizes chromosomes both in interphase and metaphase is unknown. Here we used *Xenopus* egg extract to reconstitute and image loop extrusion in single DNA molecules. We show that loops form in both metaphase and interphase, but with distinct symmetry and processivity. Condensin extrudes non-symmetric loops in metaphase, whereas cohesin extrudes symmetric loops in interphase. This work shows that loop extrusion is a general mechanism for chromosome organization, with dynamical and structural properties that are molecularly regulated to achieve different levels of chromatin organization during the cell cycle.

P1232/B360

Connecting Chromosome Structure and Dynamics through High-precision Microscopy, Genetic Perturbations and Stochastic Simulations.

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Inside cells, the DNA polymers are compacted into a spatially ordered, yet dynamic, 3D-structure. Recent genome-wide chromatin conformation capture (Hi-C) experiments reveal a hierarchical organization of the DNA structure, with DNA domains nested within bigger domains (or compartments). This structure underlies chromosomal dynamics, e.g. The probability that distant loci encounter each other, with a higher probability for intra-domain contacts relative to inter-domain contacts. To gain insight into the structure-dynamics relationship of chromatin, we combine high-precision microscopy in living *Schizosaccharomyces pombe* cells with systematic genetic perturbations and stochastic modeling of DNA looping. First, we investigate the model that the activity of SMC proteins, which play crucial roles in chromosome structure, drive chromatin dynamics by loop extrusion. Surprisingly, inhibiting the activity of SMC complexes (either condensin or cohesin) using temperature-sensitive alleles increases chromatin mobility. These results suggest that SMC complexes normally constrain rather than agitate chromatin motion. Next, we interrogate how other DNA-organizing protein complexes, including RNAP, nucleosome remodelers or histone modifiers, impact chromosome dynamics. Interestingly, we found that perturbation of the INO80 nucleosome remodeling complex, but not the SWI/SNF, RSC or FFT complexes, reduces the mobility of chromosomal loci. Taken together with prior studies in budding yeast, we suggest that INO80 uniquely couples the act of transcription and chromatin motion. Stimulated by these experimental data, we built and simulated minimalistic stochastic models that

include transcription- and nucleosome-dependent dynamics of SMC proteins and compare it to models of CTCF-dependent or random (diffusion-capture) SMC dynamics. As modeling outputs strongly depend on the parameter values, we developed a polymer-physics-inspired metric that allows a quantitative comparison of the model outputs to experimental Hi-C data. Surprisingly, a simple transcription-nucleosome-dependent model can reproduce the major features of Hi-C maps in mammalian cells without invoking positions of CTCF binding. Our work suggests that the underlying architecture of transcriptional units plays a foundational role in establishing DNA structure that is further influenced by sequence-specific factors.

P1233/B361

HP1α Is a Chromatin Crosslinker That Dictates Nuclear Mechanics and Morphology.

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The cell nucleus houses, organizes, and protects the genome. Previous findings reveal that chromatin, DNA and associated proteins, provide mechanical resistance to maintain the proper organization of the genome and shape of the nucleus to facilitate its proper function. HP1α is a known dimer that can act as a heterochromatin crosslinker, but its contribution to nuclear mechanics and morphology has been elusive. We used a novel HP1α -EGFP-AID, auxin inducible degron, cell line generated by the 4D Nucleome to degrade HP1α in 4 hours and determine its role in nuclear mechanics. Single nucleus isolation and micromanipulation force measurements reveal that HP1α is an essential mechanical component to chromatin-based short-extension nuclear force response, while not contributing to lamin A-based strain stiffening at longer extensions. As with other perturbations that decrease chromatin-based nuclear rigidity, degradation of HP1α results in abnormal nuclear morphology. Simulations of nuclear mechanics suggest HP1α could be acting either as a chromatin component or a chromatin-chromatin crosslinker but not as a lamin-chromatin crosslinker. Thus, we modulated heterochromatin levels to determine if HP1α dictates heterochromatin's mechanical rigidity to the cell nucleus. Increased heterochromatin, via the histone demethylase inhibitor methylstat, is not dependent on HP1α as it rescued both nuclear rigidity and nuclear shape in HP1α degraded nuclei. These findings suggest that even though HP1α is a heterochromatin crosslinker, HP1α crosslinking and heterochromatin provided differing contributions to nuclear rigidity and thus nucleus shape and function.

P1234/B362

DNA Methylation Status Is Sensed in the Nucleosome Self-assembly.

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We previously reported that dsDNA molecules can sense homology and those with identical sequences selectively assemble with one another. We named this phenomenon "DNA self-assembly" (1). Furthermore, we also reported that nucleosomes also have homology sensing ability and those with identical DNAs preferentially associate with one another. This phenomenon was named "nucleosome self-assembly" (2). These phenomena are facilitated or enabled by physiological concentrations of Mg^{2+} ions (1, 2). The next question to be answered is whether DNA methylation influences DNA self-assembly and/or nucleosome self-assembly. In this study, we examined the effect of DNA methylation on the

nucleosome self-assembly. Briefly, using the 601 sequence and histone octamers purified from chicken erythrocytes, homomeric or heteromeric tetranucleosomal arrays were reconstituted in *vitro. The* arrays had the 'AAAA', 'BBBB', 'AABB' and 'ABAB' structures, in which 'A' and 'B' indicate the nucleosome with unmethylated 601 and that with methylated 601, respectively. Then, using low concentrations of $MgCl_2$, these nucleosomal arrays were weakly induced to form intra-condensates with the conformation in which two nucleosomes associated and the remaining two were still free. Interestingly, in the intra-condensates, the association between the same nucleosome species was predominant, as compared to that between different species, strongly indicating that the status of nucleosomal DNA methylation is sensed in the nucleosome self-assembly. This phenomenon seems implicated in various chromatin-based biological phenomena. In this meeting, we will discuss the phenomenon we observed.
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3

Post-Transcription Gene Regulation

P1235/B363

Linking RNA Condensation to Translational Regulation.

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Post-transcriptional regulation of protein production helps maintain carefully balanced protein levels under normal growth conditions. It also allows for rapid adaptation to new environments such as the dramatic changes in the translational program associated with cellular stress responses. One means of post-transcriptional control is to change the localization or availability of mRNAs. For instance, in response to a variety of stresses, such as energy depletion or heat shock, RNA and protein are localized to biomolecular condensates termed RNA granules which are thought to be involved in translational regulation. However, the mechanisms of translational regulation during stress and the functional consequences of RNA granule formation remain unclear. In order to better understand the roles these granules and other RNA condensates play in translational regulation, we sought to determine which transcripts enter condensates in response to stress. To do so, we used ultra-centrifugation and RNA-seq to identify the transcripts which multimerize in response to heat shock in budding yeast. We find that the majority of transcripts aggregate in response to stress, while certain transcripts, such as those encoding heat shock proteins, remain soluble. Interestingly, we also observe that highly transcribed genes are more soluble than expected even under normal growth conditions. This suggests that RNA aggregation is tied to translational control at all times, not just during stress. To further investigate this phenomenon, we have designed a series of artificial reporters whose translation is regulated through a variety of mechanisms. We have used these transcripts to further explore the underlying mechanisms of RNA aggregation and its role in controlling protein production.

P1236/B364

The SUMO E3 Ligases Siz1 and Mms21 Are Required for DNA-protein Crosslink Repair.

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Accurate duplication of chromosomes and their faithful transmission to daughter cells is essential to all eukaryotic organisms. This essential process can be interrupted by DNA damage lesions including DNA-protein crosslinks (DPCs). DPCs are the covalent attachment of proteins to DNA and are physical impediments to DNA replication and transcription machinery. Failure to repair DPCs results in genomic instability, which is a hallmark of cancer. Wss1, Mre11 and Tdp1 are three DNA repair enzymes that are important for DPC repair. However, how the protease Wss1 targets DPCs for repair remains unknown. We hypothesized that for a DPC to be repaired, it must first be marked for Wss1-dependent removal. Here, we made use of an inducible Flp-step mutant to model DPC repair at a defined locus in the budding yeast *Saccharomyces cerevisiae*. Wildtype cells can tolerate expression of a single unrepairable site-specific Flp-DPC but cells deficient in Mre11 cannot. Using this approach, we screened for factors that are important for tolerance to Flp-DPC induction. We found two SUMO E3 ligases, Siz1 and Mms21, as important for tolerance to our model DPC as well as for Topoisomerase1 covalent complexes induced with Camptothecin. Mms21 functions as part of the SMC5/6 complex that is recruited to DNA double strand breaks. Our data show that mutations in the SMC5/6 complex impairs DPC repair, suggesting that DPCs are modified by Mms21 when they are converted to DNA double strand breaks. Our results provide molecular insight to how Wss1 targets DNA-protein crosslinks for removal.

P1237/B365

Mapping the Rbfox2 Interactome in Mouse Myoblasts Using a Proximity Proteomics Approach.

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Myogenesis is regulated by the coordinated expression of muscle regulatory factors that includes Mbln1, Ptbp1, Cugbp1, Cugbp2 and Rbfox2. Rbfox2 is a UGCAUG sequence-specific RNA binding protein and plays an important role in regulating alternative splicing transitions during skeletal muscle differentiation. We and others have previously reported that the Rbfox2 gene undergoes tissue-specific alternative splicing and produce multiple isoforms specific to brain, heart and skeletal muscle. However, the function of Rbfox2 isoforms and their associated protein interaction and signaling pathways in skeletal muscle differentiation are largely unknown. Thus, we used a live cell proximity-labeling proteomics approach (BioID) to identify a spectrum of new protein interactions to Rbfox2 in C2C12 myoblast cells. This technique is particularly useful in identifying weak or transient interactions in living cells that are not detected by co-immunoprecipitation or yeast two-hybrid systems. In this study, we used three different Rbfox2 isoforms either containing ubiquitously expressed (B40) or a muscle specific (M43) exon or lacking an RNA recognition motif. The BioID fusion Rbfox2 isoforms are predominantly localized to the nucleus of the cell, and regulates the alternative splicing of an exogenously expressed Non-muscle Myosin Heavy Chain IIB mini-gene, reflecting native Rbfox2 function and subcellular distribution. To identify Rbfox2-proximal proteins in living cells, biotin was added to the cells expressing myc-BirA*Rbfox2 or myc-BirA*control followed by streptavidin pull-down and mass spectrometry analysis. Following mass spectrometry analysis, we filtered the data for a minimum fold change of >3 over control. This analysis identified 453 proteins proximal to at least one Rbfox2 isoform and also

identified known interactors of Rbfox2 such as Ataxin2. Of the 453 total proteins identified, 94 were common to all three Rbfox2 isoforms. This common set of 94 proteins was highly enriched for gene ontology terms related to the spliceosomal complex assembly, N6-methyladenosine-containing RNA binding and histone deacetylase binding that suggests a novel physiological role for Rbfox2.

P1238/B366

Terminal Uridyltransferase 7 Regulates Toll-like Receptor 4-driven Inflammatory Response by Controlling Messenger RNA Stability.

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Innate immune responses are the first line of host defense to effectively clear pathogens and repair damaged tissues, however, dysregulation of inflammatory responses has been associated with numerous illnesses, such as cancer, sepsis, and metabolic disorders. Therefore, the innate immune responses must be tightly regulated. Toll-like receptors (TLRs) play a major role in the innate immune-mediated responses by sensing pathogen-associated molecular patterns from pathogens or damage-associated molecular patterns released from stressed or damaged cells. Various mechanisms have been reported to regulate TLRs-driven immune responses, including phosphorylation, ubiquitination/deubiquitination, and transcription/post-transcriptional control. Among them, post-transcriptional regulation is a rapid and flexible mechanism to modulate the expression of inflammatory-related genes. However, the molecular mechanism of post-transcriptional regulation still remains largely unknown. We previously studied the regulation of TLRs-mediated immune responses, and found a terminal uridyltransferase (TUT), TUT7, which was induced after TLR ligands treatment, including Pam_3CSK_4 (TLR1/2), poly(I:C) (TLR3), LPS (TLR4) and CpG-1826 (TLR9). TUTs are known to have the ability to uridylate or adenylate small RNAs and mRNAs, thereby modulating the stability of the target RNAs. Nevertheless, the function of TUT7 in TLRs-induced immune response remains to be discovered. Here, we demonstrate that depletion of TUT7 in both bone marrow-derived macrophages (BMDMs) and RAW264.7 macrophage cells results in dysregulated TLR4-triggered cytokine expression, including decreased the production of pro-inflammatory cytokines IL-1β, IL-6, and IL-12. Downregulation of IL-6 mRNA expression is also observed in TUT7-deleted BMDMs treated with various TLR agonists. We further reveal that TUT7 associates with and uridylates the endonuclease Regnase-1 mRNA, resulting in destabilization of Regnase-1 mRNA, which leads to upregulated expression of its target gene IL-6. Collectively, our findings indicate that TUT7 is involved in the regulation of TLR4-triggered immune responses by modulating mRNA stability of distinct inflammatory mediators.

P1239/B367

Hypoxia-inducible Factors Repress Translation Via Alkbh5-mediated M^{6}A RNA Demethylation in Hypoxic Breast Cancer Cells.

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Hypoxia-inducible factors repress translation via ALKBH5-mediated m^{6}A mRNA demethylation in hypoxic breast cancer cells *N*^{6}-methyladenosine (m^{6}A) is a post-transcriptional modification of mRNA that alters mRNA and protein expression with emerging roles in cancer. Intratumoral hypoxia is a driving force for cancer progression, and expression of the m^{6}A demethylase ALKBH5 is strongly induced by hypoxia-inducible factors (HIFs), leading to global changes to the m^{6}A landscape in hypoxic cancer cells.

However, the relationship between transcriptional and post-transcriptional regulation by HIFs and ALKBH5, respectively, and downstream functional consequences of global changes in m^6A have not been examined. In this study we used total RNA-sequencing, m^6A sequencing, and mass spectrometry proteomic analysis to elucidate the shared and distinct roles of HIF and ALKBH5 in mediating breast cancer cell response to hypoxia, and how this is mechanistically accomplished through m^6A. We report that in hypoxic MCF7 breast cancer cells the majority of HIF-dependent genes are also ALKBH5 dependent at both the mRNA and protein levels. Additionally, the repression of cell cycle-promoting genes is both HIF- and ALKBH5- dependent, and knockdown of HIF-1α/HIF-2α or ALKBH5 causes increased levels of cancer cell proliferation in hypoxia. Loss of either HIF-1α/HIF-2α or ALKBH5 expression also causes increased levels of m^6A^+ RNA. Through m^6A-RNA sequencing we found that transcripts that promote translation initiation are consistently methylated in control cells, while transcripts encoding proteins mediating oxidative metabolism are dynamically demethylated in response to hypoxia. Loss of HIF-1α/HIF-2α or ALKBH5 broadly alters m^6A coverage patterns on mRNA, and specifically prevents demethylation of oxidative metabolism gene transcripts, which impairs the switch from aerobic to glycolytic metabolism in hypoxia. We also found that the most highly expressed m^6A reader protein in MCF7 cells is YTHDF1, which promotes translation of m^6A-modified mRNA. Indeed, m^6A^+ mRNAs produce significantly more protein in response to hypoxia than do m^6A^- mRNAs. Therefore, HIFs and ALKBH5 work in concert to limit translation, restrict cancer cell proliferation, and upregulate glycolytic metabolism in response to hypoxia.

P1240/B368

A Genome-wide Crispr-cas9 Knockout Screen in Human Cells to Identify New NMD Factors and Regulators.

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Nonsense mediated RNA decay (NMD) is a highly conserved RNA surveillance and gene regulation mechanism in eukaryotes. NMD degrades nonsense RNA transcripts containing premature translation termination codons (PTCs), and down-regulates other non-mutant RNAs containing other features on the RNA, such as upstream open reading frames, inclusion of PTCs-containing exons, introns in 3' untranslated region (UTR), and long 3' UTRs. Therefore, NMD is important for normal physiology and in diseases, where it modifies the phenotypic outcomes of many genetic disorders and cancers caused by nonsense mutations. The known core NMD factors, including UP-Frameshift (UPF) proteins and suppressors with morphogenetic defects in genitalia (SMG) proteins were identified in *S.cerevisiae and C. elegans.* While the NMD pathway is conserved in eukaryotes, there are significant differences in the mechanisms of NMD across species. Strikingly, several branches of NMD have been observed in humans, where NMD can occur in the absence of some core NMD factors, such as UPF2, or UPF3B, or in the absence of exon junction complexes that are known to strongly promote NMD in humans. Therefore, to fully dissect the mechanisms of NMD in humans, an unbiased approach is required to identify factors involved in NMD. We have developed a novel NMD reporter system to effectively measure NMD in human cells and used it to performed a genome-wide CRISPR/Cas9 knockout screen to identify genes important for NMD. Among our top validated hits, we have identified 9 novel genes that promote NMD in human cells. Our screen also identified SF3B complex of the spliceosome as one of the most enriched complexes, and additional experiments uncovered a previously unrecognized role of SF3B in NMD. Uncovering how the novel genes and protein complexes function in NMD will provide us with a better understanding of NMD in humans.

P1241/B369

Translational Regulation of the Antiapoptotic Protein Survivin by Control Elements in the 5' Untranslated Region.

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Survivin is an anti-apoptotic protein that is ubiquitously expressed during embryonic development but is absent in most adult tissues, with exceptions, including the gastrointestinal epithelium. Despite its relevance in physiological processes, Survivin is re- or over-expressed in most cancer types correlating inversely with patient survival. The most important mechanisms studied related to the control of Survivin expression include transcriptional regulation, protein stability, and post-translational modifications, however to date, translational control has been poorly studied. Thus, we focused particularly on the translational control elements present in Survivin 5' untranslated region (5'UTR). Bioinformatic analysis of ribosome occupancy on Survivin 5'UTR (GWIPS online server) showed the presence of elongating ribosomes upstream the canonical initiator AUG, opening the possibility of the presence of an alternative upstream initiator AUG (uAUG). As expected, this uAUG was found at position -71, however, its impact on Survivin translation has not been determined. This study employed mainly human gastric (AGS and Ges-1), embryonic (HEK293T) and cervical (Hela) cell lines. The activity of the putative uAUG was assessed by Renilla reporter assay, qRT-PCR, Western blot, *in vitro* transcription/translation and DNA recombinant techniques (DNA cloning and point mutations). Results: Transfection of Renilla reporter plasmids under the control of CMV promoter, showed that when the Survivin 5'UTR was cloned upstream of the Renilla gene, the expression of the reporter gene was decreased without changes in its mRNA levels; however, when the uAUG was mutated to AUU, the inhibitory effect was abolished. Since this uAUG was not positioned in frame with the Survivin initiator AUG, we inserted nucleotides in two different positions between both AUGs (In-Frame reporter constructs), which also increased reporter activity. Moreover, immunodetection of Renilla (35 kDa) by Western blot revealed the presence of a second band (38 kDa approximately) in cells transfected with these In-frame reporter constructs, indicating that the uAUG was actually functional in all cell lines tested. In conclusion, our experimental data demonstrate the presence of an alternative and inhibitory initiator uAUG in the Survivin 5' UTR. This inhibitory uAUG could help to understand how Survivin expression is downregulated under stress conditions. Funding: This work was supported by the grants Fondecyt 11711715 and CIP16020 (MAVV).

P1242/B370

Engineering Combinatorial and Dynamic Decoders Using Synthetic Immediate-early Genes.

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Mammalian cells use signaling pathways to process information about the outside world, and then decode pathway activity into downstream gene expression. Some hallmarks of this decoding process are dynamic control, where only certain time-varying signals trigger a response, and combinatorial control, where two signals are interpreted together through a logic gate. While these hallmarks are known, their application to the process of gene expression remains unclear. One hypothesis for how cells apply these principles stems from a common design principle of gene expression, which is regulation throughout

multiple steps of the central dogma. In addition to transcriptional activation, signaling pathways can modulate mRNA stability, protein translation, or protein stability. However, the signal-processing properties of this multi-step regulation are still poorly understood. Here, we have developed a flexible platform to produce synthetic immediate-early genes (synIEGs) that contain user-defined transcriptional and post-transcriptional regulation, as well as live-cell biosensors throughout the central dogma. First we found that synIEGs can be randomly integrated into the genome and transcribe on the same timescale as endogenous immediate-early genes, suggesting that, for this class of genes, the specific location in which we integrate the genes may not matter. We next demonstrate the utility of this approach to study the signaling pathway to gene expression interface by engineering synIEGs that implement dynamic or combinatorial control in mammalian cells. Our synIEGs demonstrate that regulation at multiple nodes through the central dogma, along with the modular plasticity of mRNA/protein domains, can be used to decode both dynamic and combinatorial information. We believe that, in the future, this approach will allow us to better understand how genes interpret signaling pathway activity as well as engineer genes that respond to user-defined inputs.

P1243/B371

Meiotic Production of N-terminally Truncated Proteins in *S. Cerevisiae*.

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Budding yeast meiosis is an intricate developmental program that requires highly dynamic and coordinated gene expression regulation. We used start-site ribosome profiling to identify translation start-sites genome-wide across meiosis, providing a view of translation across a unperturbed developmental process. These data are particularly useful for identifying ORFs originating from within canonical full-length proteins, including those that initiate from downstream start sites that are in-frame with the canonical proteins and end at the same stop codon (N-terminal truncations). We have identified hundreds of cases of these truncated proteins, many of which are dynamically regulated across meiosis. In addition, we have begun molecular characterization of examples of truncated proteins to determine functional relevance during both meiosis and vegetative growth. Our work points towards potential functional roles for previously uncharacterized truncated proteins and suggests a greater diversity of gene products produced even within compact genomes.

P1244/B372

Tumor Regulator PICT1 Interacts with MTR4/exosome and Is Involved in the Pre-rRNA Processing.

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RNA exosome is a 3’-5’ exonuclease complex, which is involved in processing and degradation of a wide variety of nuclear RNAs by collaborating with an RNA helicase MTR4. During ribosome biogenesis, MTR4/exosome plays critical roles in the processing of pre-RNAs. In *Saccharomyces cerevisiae*, Nop53 has been identified as an adaptor that recruits Mtr4 to the 3’-processing site of 5.8S rRNA. Intriguingly, PICT1, a putative mammalian ortholog of Nop53, has been characterized as a nucleolar localized tumor-regulating protein, which controls cell proliferation through MDM2-p53 axis. To examine a possible role of PICT1 in the connection between ribosome biogenesis and cell proliferation process, we explored the function of human PICT1 in the pre-rRNA processing during ribosome biogenesis. Physical interaction between PICT1 and MTR4/exosome was confirmed by co-immunoprecipitation, which depends on the

integrity of conserved arch interacting motif (AIM) in PICT1. Knockdown of PICT1 led to a defect in pre-rRNA cleavage as shown by accumulation of the 32S processing intermediate. On the contrary, overexpression of AIM-defective mutants of PICT1 led to an accumulation of 12S processing intermediate, showing inhibition of MTR4/exosome-mediated processing step of 12S rRNA. These results suggest that PICT1 functions at the two distinct steps during pre-rRNA processing. Furthermore, knockdown of either PICT1 or MTR4 led to upregulation of p53 in cells, although knockdown of DIS3 or RRP6, catalytic subunits of the nuclear exosome, did not affect the p53 levels. Thus, these results suggest that interaction between MTR4 and PICT1, but not 12S pre-rRNA processing, might be important for transmitting the nucleolar stress to the p53 regulation.

P1245/B373

Autoregulation of Hoxa1 during Mouse Es Cell Differentiation.

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Autoregulation of Hoxa1 during Mouse ES Cell Differentiation Emelogu Ugochi and Eduardo Martinez-Ceballos, Southern University and a & M College Baton Rouge, LA Hoxa1, a member of the homeobox (Hox) family of transcription factors, is an important regulator of embryonic patterning and organogenesis. Inactivation of both alleles of the *Hoxa1* gene in mice results in numerous developmental defects, including hindbrain deficiencies and abnormal skull ossification, and ultimately, in neonatal death. In humans, mutations in the *HOXA1* gene have been described in association with various CNS disorders. However, in spite of the recognized importance of Hoxa1 during vertebrate development, little is known about its molecular mechanism of action in cells and tissues. The goal of this work is to shed light on the molecular mechanisms of Hoxa1 transcriptional gene regulation and function. By performing preliminary ChIP-on-chip analyses, we have identified a putative Hoxa1 binding site in the *Hoxa1* coding region. As overexpression of Hoxa1 in mouse ES cells was found to inversely correlate with the endogenous expression of *Hoxa1*, we hypothesize that the Hoxa1 protein can repress its own gene expression by directly binding to the *Hoxa1* coding region. Preliminary in vitro DNA binding/EMSA assays using biotinylated DNA probes, that correspond to different regions of the *Hoxa1* coding region, resulted in bound DNA observed when nuclear extracts from Retinoic Acid-treated J1 ES cells were used but not with extracts from untreated J1 ES cells. This band was not observed when 200-fold excess unlabeled DNA (specific competitor) was added. All together, our studies identified a putative Hoxa1 binding site in the *Hoxa1* coding region, which suggests the presence of a novel autoregulatory mechanism for this transcription factor. The results from this project will lead to the elucidation of the specific roles played by Hoxa1 on the differentiation of ES cells, which will represent a significant contribution to the area of stem cell biology.

P1246/B374

Brct Domains Contain an Intrinsic Post-translational Modification (ptm) Recognition Code That Affects It Stability.

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BRCA1 C-terminal (BRCT) domains are protein binding modules that relay signals throughout various cellular pathways. The human genome encodes 23 BRCT-domain containing proteins that are commonly found in tandem and have been characterized as phosphoprotein binding domains with functions throughout the DNA damage response. Emerging evidence suggests these domains bind other post-

translationally modified (PTM) substrates although the details are not defined. Recently, the BRCT domains from BRCA1 were shown to bind an asymmetrically dimethylated arginine (ADMA) p300 substrate to regulate the transcription of the p21 in response to DNA damage. Based on this observation, we hypothesize that the methyl-specific binding site for the ADMA interaction is distinct from the phospho-substrate recognition interface. To test this hypothesis, we conducted peptide binding and thermal stability experiments to demonstrate the change in stability upon binding phospho- versus methylated substrates. Our results show that phosphorylated substrates increase the stability of BRCA1's BRCT domains. Doubly phosphorylated substrates showed the greatest increase in stability while the methylated substrate demonstrated a decrease in stability. To define the BRCT-ADMA binding interface, we set out to determine the three-dimensional structure. High-throughput co-crystallization of the BRCA1 BRCT protein with a ADMA peptide derived from the p300 substrate identified one condition for crystal growth optimization. In silico modeling of the BRCA1 BRCT structure with the ADMA molecule has highlighted two potential binding sites for the ADMA substrate, which is not conserved throughout the BRCT domain family. Our results indicate that while BRCT domains share a conserved mode of binding phosphorylated substrates, which increases their stability, and their ability to binding methylated substrates may not be conserved. These finding will be critical in understanding how BRCT domains can accommodate binding various modified substrates and regulating signals throughout the DNA damage response.

P1247/B375

Characterizing Alternative Splicing Dynamics of Calcium Channel $Ca_v1.3$.

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L-type voltage-gated calcium channel $Ca_v1.3$ contains a pore-forming 1αsubunit that is encoded by *CACNA1D*, a gene which has been shown to undergo alternative splicing. Mutations in *CACNA1D* have been associated with neurological diseases such as spontaneous seizure disorder and epilepsy. Previous studies have shown that alternative splicing of the C-terminus of $Ca_v1.3$ results in a truncated variant of exon 42 (E42) that, when compared to the full-length exon, lowers the voltage threshold needed to activate the calcium channel. A lower voltage threshold of calcium channels increases neural excitability and therefore has many clinical implications. Previous studies have also linked the alternatively spliced N-terminus of $Ca_v1.3$ to autism spectrum disorder. In a human patient study, a naturally occurring missense mutation in both mutually exclusive exons 8a and 8b has been correlated with autism spectrum disorder. These results highlight the important role that alternative splicing plays in $Ca_v1.3$ function. We were able to confirm four alternatively spliced regions, E8-E11, E12-E15, E32-36, and E46-E48 in *Cacna1D*. By semi-quantitative RT-PCR analysis, we found that these alternatively spliced variants are expressed at higher levels in brain-derived tissues when compared to non-brain derived tissues. We were also able to construct minigenes of E9-E10, E13-14, E33-E34, E35, and E47 to investigate the regulatory mechanisms of alternative splicing of *Cacna1D*. While alternative splicing of $Ca_v1.3$ has been shown to play important roles in electrophysiological properties and neurological disorders, the dynamics of alternative splicing of $Ca_v1.3$ remain poorly understood. Our results serve to characterize the alternative splicing dynamics in *Cacna1D* for future studies aimed at determining whether or not certain splice isoforms can lead to changes in the channel's electrophysiological properties.

P1248/B376

Stop Codon Context Influences Quality Control of Truncated Proteins.

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Cells protect themselves from the deleterious effects of truncated protein products via multiple quality control pathways. Nonsense-mediated mRNA decay (NMD) is one such mechanism that degrades mRNAs containing premature termination codons in order to prevent aberrant protein production. While much is known about NMD, mechanisms that confer variability in NMD efficiency, and how cells handle truncated proteins produced in such scenarios, are poorly understood. Here, we utilize human genetic data and cell-based reporter assays to investigate whether the sequence context of a premature stop codon influences the fate of the truncated protein product. Sequence analysis of nonsense variants found in the healthy human population (gnomAD database) shows a high degree of enrichment for glycine codons at the -1 position of the premature stop codon, suggesting that it is a more permissive context for nonsense mutations through the course of evolution. Consistent with a recent report of C-terminal glycine acting as a degron, reporter assays show that Gly|PTC dicodon context induces down-regulation of the truncated proteins, but only in the context of premature translation termination. Moreover, the identity of the -1 codon and the premature termination codon together influence the level of the premature protein product. Based on these results, we conclude that the concerted action of protein and RNA quality control acts as a failsafe mechanism to limit truncated protein products in a sequence-dependent manner.

4

Nuclear Pore Complexes

P1249/B378

Disassembly and Reassembly of the Nuclear Pore Complex in C9orf72 Als/ftd.

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Nucleocytoplasmic transport, controlled by the nuclear pore complex, has recently emerged as a pathomechanism underlying neurodegenerative diseases including C9orf72 ALS/FTD. However, little is known about the underlying molecular events. Using super resolution structured illumination microscopy of twenty three nucleoporins in nuclei from C9orf72 iPSC derived neurons and postmortem human motor cortex we identify a unique subset of eight nucleoporins lost from the neuronal nuclear pore complex. POM121, an integral scaffolding nucleoporin, appears to coordinate the disassembly and reassembly of the nuclear pore complex in human neurons impacting nucleocytoplasmic transport, and subsequent cellular toxicity in C9orf72 iPSNs. These data suggest that POM121 is an integral nucleoporin in the maintenance of the nuclear pore in human neurons and loss of POM121 from the nuclear pore complex, as a result of expanded C9orf72 ALS/FTD repeat RNA, initiates a pathological cascade affecting nuclear pore complex integrity, function, and overall neuronal survival.

P1250/B379

Nuclear Pore Complexes Assemble from Nucleoporin Condensates during Drosophila Oogenesis.

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Nuclear Pore Complexes (NPC) span the nuclear envelope (NE) and allow transport between the cytoplasm and the nucleus. In gametes and early embryos NPCs are also abundant at the ER in specialized compartments termed an nulate Lamellae (AL). While two mechanisms have been conceptualized to explain how NPCs insert to the NE during mitosis and interphase respectively it is unknown how NPCs assemble in the absence of the nucleus as spatial cue. We address this question in vivo during oogenesis in *Drosophila* and suggest a third, non-canonical NPC assembly pathway. We show that in the germline Nucleoporins (Nups) form granules that display hallmarks of phase separated biocondensates. They are NPC precursors that interact to exchange Nucleoporin material for NPC biogenesis along the ER. Different from canonical NPC assembly, the Nucleoporin Nup358 appears key for this process: Regulated by the small GTPase Ran and the nuclear transport receptor Crm1 it condenses into NPC assembly platforms that in turn control pore biogenesis in a Ran and Microtubule dependent manner. We show that Nup358 condensation is necessary for NPC assembly that cannot proceed from the soluble Nucleoporin. We delineate a third previously uncharacterized NPC assembly pathway that operates from Nucleoporin condensates away from the nucleus and under conditions of cell cycle arrest.

P1251/B380

TorsinA and Neuronal Nuclear Pore Complex Biogenesis.

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TorsinA is a AAA+ protein localized to the endoplasmic reticular (ER)/nuclear envelope (NE) endomembrane space. A loss-of-function mutation in the gene encoding torsinA causes DYT1 dystonia, a neurodevelopmental movement disorder. Yet, the biological function of torsinA remains largely unknown and the molecular defects underlying DYT1 dystonia remain poorly understood. In mouse primary neurons, we find a dramatic upregulation in nuclear pore complex (NPC) biogenesis during neuronal maturation. In contrast to wild-type neurons, torsinA-null neurons develop increasingly mislocalized clusters of NPCs. These clusters appear to represent halted intermediate states of NPC assembly, as they contain early- but not late-recruited NPC components. Consistent with this notion, torsinA-null neurons exhibit defects in NPC-mediated nucleocytoplasmic transport. Interestingly, NPC density is unaffected in torsinA-null neurons, suggesting that torsinA helps maintain NPC localization and assembly, but not number. Similar to prior findings in *vivo*, primary cultures of torsinA-null neurons develop evaginations of the inner nuclear membrane (NE buds). The emergence of NE buds coincides with the formation of mislocalized NPC clusters, implicating an association between these events. Considered together, our findings suggest that torsinA plays previously unidentified roles in the localization and assembly of new NPCs during a key period of neuronal development, implicating aberrant NPC biogenesis in the pathogenesis of DYT1 dystonia.

P1252/B381

Oligodendrocyte Nuclear Pore Biology in Health and Disease.

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Laminopathies are a rare but devastating group of genetic disorders resulting in part from mutations in genes encoding lamin proteins. Lamins are intermediate filament proteins lining the inner nuclear membrane that facilitate structural integrity, genome organization, and arrangement of nuclear pore complexes (NPCs). Mammalian B-type lamins are ubiquitously distributed in the body, and a tandem gene duplication in *LMNB1*results in autosomal dominant leukodystrophy with autonomic disease (ADLD). ADLD is a fatal, degenerative demyelinating disease, with symmetrical demyelination beginning in the 4thor 5thdecade of life. While it appears that oligodendrocytes are particularly sensitive to increased *LMNB1* dosage, the nature of this vulnerability, and the effect of the mutation on oligodendrocyte progenitor cells (OPCs) have not been elucidated. This work aims to define how LMNBI changes the nuclear biology of oligodendrocyte lineage cells over the course of their differentiation, and examine how these cells are affected in the context of ADLD. Preliminary evidence from our lab and others shows that oligodendrocytes undergo dramatic changes in nuclear architecture (including arrangement of nuclear pore complexes, NPCs) and heterochromatin distribution over the course of differentiation. We have determined that mature oligodendrocytes have significantly reduced nuclear occupancy of multiple components of the NPC in comparison to neurons and astrocytes, suggesting that oligodendrocytes might be especially sensitive to perturbations in the ratio of these or other nuclear proteins. Additionally, we observe differences in expression of laminB1 at the protein level between gray and white matter oligodendrocytes in wild-type mice, with reduced immunoreactivity of laminB1 specifically in white matter oligodendrocytes. To define nuclear pore alterations in oligodendrocytes, we generated *LaminB1^{BAC}; MOBP-eGFP* mice in which laminB1 is over-expressed and all oligodendrocytes are fluorescent. Consistent with the late adult-onset nature of the disorder, we do not observe pathological signs in *vivo* or nuclear abnormalities in oligodendrocyte nuclei isolated from the brains of 2 month-old mice. Aging studies of these mice are currently in progress. To better mimic the human disorder, we describe a strategy to generate a new mouse model of ADLD using a dual-gRNA system for CRISPR-Cas9, a strategy which has been previously used to generate large structural variations in the genome. Together, these experiments add to the expanding biology of nuclear proteins in cell and tissue-specific disorders, and shed light on disease-relevant pathways in ADLD.

P1253/B382

Nuclear Envelope Dynamics in Torsin-deficient Cells in Relation to Nuclear Pore Biogenesis.

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Torsin ATPases are widely conserved, essential AAA+ ATPases residing in the nuclear envelope (NE) and endoplasmic reticulum. The molecular function(s) of Torsins remain largely elusive. A shared phenotypic hallmark observed upon Torsin manipulation in metazoan organisms and numerous tissue culture models is the presence of omega-shaped herniations in the NE. Both the molecular composition and the dynamics of these herniations are largely unknown. We identified FG-nucleoporins at the base of the

omega-shaped herniations (also known as “blebs”), which has a diameter comparable to that of the nuclear pore complex (NPC). NUP358, an NPC component that is added late during NPC assembly, is underrepresented at these sites. A comparative proteomics approach revealed myeloid leukemia factor 2 (MLF2) as an overrepresented constituent of NE fractions from Torsin-deficient cells relative to those of wild type cells. MLF2 specifically localizes to the bleb lumen, while model substrates of canonical nuclear transport (NLS-GFP/NES-GFP) appear to be excluded. Engineered variants of MLF2 can now be utilized as molecular probes to study bleb formation in a variety of experimental settings. MLF2-GFP, for example, represents the first bleb-specific live cell imaging marker, revealing unanticipated, cell cycle-dependent dynamics via lattice light sheet microscopy. Blebs resolve during mitosis, and reform rapidly and synchronously within a short period after NE reformation. Based on the observed kinetics of bleb formation, and their strict dependence on Pom121-an NPC component previously implicated in interphase NPC biogenesis- we suggest a role for Torsins during NPC assembly.

P1254/B383

Role of Retinoblastoma on Nup98-associated Leukemia.

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NUP98-associated leukemia is rare but recurring and characterized by high aggressiveness and poor treatment outcome. NUP98 chromosomal translocations are characterized by overexpression of HOXA clustered genes. HOXA gene over-expression, often in association with the HOX-binding partner MEIS1, contributes to the leukemogenic process, namely, to failed differentiation and accumulation of myeloid blasts. The nuclear envelope (NE) phenotype of NUP98-associated leukemia is correlated with nuclear lobulations and alterations in the nuclear lamina and the lamina associated protein 2 α (LAP2α). LAP2α is an important regulator of the tumor suppressor **retinoblastoma protein** (pRb), which is a known regulator of epigenetic modifications associated with HOXA gene transcriptional status. The aim of this research project is to study the impact of pRb on NE phenotype of NUP98-associated leukemia, additionally the **epigenetic landscape** that regulates HOXA gene expression associated to NUP98 fusion proteins. The NE-associated proteins, as lamin A, lamin B and LAP2α were studied by confocal laser scanning microscopy and electron microscopy in HeLa cells, mouse bone marrow cells expressing NUP98 fusion proteins and in leukemic blasts derived from patients. The NE components were analyzed upon depletion of retinoblastoma. To address the epigenetic modifications, silence and active histone markers were evaluated by quantitative analysis providing an overview of specific histone patterns. We observed that NUP98 fusion proteins induced aberrant NE phenotype and the alterations in NE morphology are dependent on pRb. Co-localization coefficients analysis between NUP98 chimeras and distinct histones marks suggests that NUP98 chimeras colocalize with specific active and repressive sites, trimethylation of lysine 4 on histone H3 (H3K4me2) and trimethylation of lysine 27 on histone H3 (H3K27me3), respectively. Leukemogenic NUP98-fusions cause diverse perturbations in NE organization which are dependent on pRb pathway. Our results unveil a novel role for pRb in AML and may suggest a potential role in NUP98-related leukemia. Furthermore, our data reveal that specific epigenetic modifications co-localize with NUP98 fusion protein, promoting active and repressive transcription. We could focus now on a properly targeted specific locus, HOXA9, deployed a dead RNA-guided nuclease Cas9 to the engineered peroxidase APEX2 and delineate the complex molecular mechanism associated to pRb.

P1255/B384

Basket Nucleoporin Tpr Is an Integral Component of Transcription and Export 2 (TREX2) RNA Export Pathway.

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Exchange of molecules between the cytoplasm and the nucleus occurs through conduits called nuclear pore complexes (NPCs). NPCs consist of roughly 30 distinct proteins (nucleoporins), forming a central channel with filaments extending into the nucleus and cytoplasm. Basket nucleoporins (Nup153, Tpr, and Nup50) localize to nucleoplasmic filaments and regulate many processes including nucleocytoplasmic trafficking, protein modification, chromatin remodeling and transcription, as well as mRNA processing and export. Moreover, the nuclear basket works as a docking platform for other proteins, including transport receptors and subunits of the TRanscription and EXport 2 (TREX-2) complex. Despite numerous studies, the structural organization of basket nucleoporins within the basket and their interaction with transcription and export machinery is still controversial. In addition, understanding the basket nucleoporins roles in vertebrate cells is complicated because their depletion by RNAi requires an extended incubation, during which all processes may be increasingly disrupted, resulting in highly pleiotropic phenotypes, many of which are secondary consequences of nucleoporin loss. To distinguish the individual functions of nucleoporins, we used CRISPR/Cas9 to biallelically insert auxin-inducible degrons (AID) tags into nucleoporin genes. Auxin addition caused complete degradation of the targeted nucleoporins within an hour; the loss of either Nup153 or Tpr caused growth arrest and eventual cell death within next 24 hours. Acute basket nucleoporin depletion did not destabilize other domains of the nuclear pore, nor did it cause nuclear envelope deformation. The degradation of any individual basket nucleoporin did not disperse either of the other two from assembled nuclear pores, except for Nup50 dispersion after Nup153 depletion. Given the minimal structural perturbations that we observed in the absence of these nucleoporins, we tested whether their loss causes defects in RNA abundance and localization. We found that loss of individual nucleoporins caused dramatically different impacts on transcriptome. Importantly, Tpr elimination caused rapid and profound changes in transcriptomic profiles within two hours of auxin addition with the retention of RNAs within the nucleus. These changes were dissimilar to shifts observed after loss of Nup153 or Nup50, but closely related to changes after depletion of transport factor NXF1 or the GANP subunit of the TRanscription-EXport-2 (TREX-2) mRNA export complex. Moreover, the NPC association of GANP was specifically disrupted upon Tpr loss. Together, our findings indicate a singular and pivotal role of Tpr in regulating gene expression through TREX-2-dependent mRNA export.

P1256/B385

Nup98-dependent Transcriptional Memory Is Established Independently of Transcription.

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Nuclear genome architecture is achieved in part through interactions between individual chromosomal regions and nuclear scaffolds. One such nuclear scaffold is the nuclear pore complex (NPC), which is embedded in the nuclear envelope, and consists of approximately 30 different subunits, called nucleoporins (Nups). In addition to its nuclear transport function, Nups can interact with underlying chromatin, specifically at regulatory DNA elements, including promoters and enhancers. Strikingly, we

discovered that the NPC component Nup98 facilitates the looping contacts between promoters and enhancers, revealing Nups as a new class of architectural proteins that organize the 3D architecture of the genome and influence gene regulation. Consistent with this idea, we characterized the role of Nups in developmental transcriptional memory, in which ecdysone hormone-induced genes are activated more robustly in cells that have previously experienced the presence of the hormone. Interestingly, loss of Nup98 does not affect transcription during initial induction, but results in slower activation during re-induction, demonstrating a loss of transcriptional memory. Using single-molecule RNA FISH to measure activation dynamics of ecdysone-induced genes and mathematical modeling we found that the stablishment of transcriptional memory is independent of active transcription during initial induction but instead, relies on the length of time between hormone treatments.

P1257/B386

In *Cellulo* Structure of the Nuclear Pore Complex and Its Implications in Mrna Export and Nuclear Pore Turnover by Autophagy.

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The nuclear pore complex (NPC) is an essential membrane protein complex in eukaryotes shaping a hole in the nuclear envelope. Due to its dimensions (~100 MDa in *H. sapiens*) the elected method to determine the structure of the intact complex scaffold has been cryo electron tomography and subtomogram averaging (vonAppen, 2016) in combination with crystal structures from purified components (Lin, 2019). In addition, efforts to investigate the full NPC structure extracting it from its double membrane context have produced two structures in *S. cerevisiae* (Yang, 1988; Kim, 2018). In our study we perform extensive cryo-FIB-milling coupled to cryo-electron tomography and subtomogram averaging to get structures of the entire NPC scaffold in exponentially-growing cells with and without protein knock-outs. The new structures not only largely overcomes in resolution previously determined structures with the same method (Mahamid, 2016; Mosalaganti, 2018), but also shows significant differences in sub-complexes architecture to previous in *vitro* work (Kim, 2018), emphasizing the importance of determining structures of membrane proteins in their native membrane context. We combine the in *cellulo* structures with integrative modeling and crosslinks analysis: Y-complexes show a very extended conformation in comparison to previous studies and we propose a new model that takes into account our in *cell* conformation. In addition, the main mRNA export platform architecture reveals a new configuration that accommodates spatially and temporally the existent biochemical data with the release of transport factors occurring in the cytoplasm. Shifting a Nup116 knock-out strain to not-permissive temperature, we activate the formation of *herniae* (Wente, 1993), protrusions of the outer and inner nuclear membrane forming evaginations conserved in human cells and linked to Dystonia (Laudermilch, 2016). Milling this knock-out strain let us discover that at the basis of the *herniae* there is a partially assembled NPC, solving an outstanding conundrum in literature. The new mRNA export architecture discloses also the cytoplasmic exposure of an NPC-intrinsic autophagic receptor (Lee et al, submitted). We validate the NPC-Atg interaction obtaining high-resolution snapshots using correlative light and electron microscopy on plastic sections (Kukulski, 2011), conventional electron microscopy and 3D-cryo CLEM (Arnold, 2016) demonstrating that NPC degradation occurs through complex membrane remodeling events at the nuclear envelope and passes through an autophagosome intermediate owning

NPC-containing nuclear vesicles in the cytoplasm. Our results highlight the power of in *cell* structural biology to provide novel insights into two fundamental processes of eukaryotic life.

P1258/B387

Characterizing Nuclear Pore Complex Heterogeneity Using Quantitative Super-resolution Microscopy.

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Nuclear pore complexes (NPCs) span the inner and outer nuclear membranes and facilitate the transport of macromolecules across the nuclear envelope. NPCs also have transport independent functions, including regulating gene expression and chromatin organization, influencing nuclear envelope integrity and remodeling, and acting as signaling hubs at the nuclear periphery for proteins and complexes involved in DNA repair and protein homeostasis. While the NPC structure is largely conserved throughout eukaryotes, recent studies have found that NPC number, distribution and composition varies across cell types and can change dramatically in multiple disease states and throughout development. Despite these findings, the mechanisms used to regulate and alter NPC number and composition, as well as the functional consequences of NPC heterogeneity remain unclear. To address these questions, we developed quantitative microscopy and image analysis tools to determine NPC number, distribution and composition in the fission yeast *Schizosaccharomyces pombe*. First, we used three-dimensional structured illumination microscopy (3D-SIM) to provide the first quantitative description of NPC number and distribution in *S. pombe*. Additionally, by combining 3D-SIM with single-particle averaging we identified a region of the nuclear envelope near the spindle pole bodies that contains NPCs lacking a specific subset of NPC components. We are currently investigating how this pool of specialized NPCs is established and maintained, and whether the heterogenous NPC composition in this region is required for nuclear envelop remodeling events that occur during spindle pole body insertion.

P1259/B388

Elys and Nup153 an chor the Nuclear Pore Complex to Nuclear Lamins.

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The nucleoporins Elys and Nup153 are localized on the nucleoplasm side of the nuclear pore complex (NPC) where they contribute to the structure of the nucleoplasmic ring and basket of the NPC and may thus interact with the nuclear lamina (NL). The NL contains type-V intermediate filament proteins that assemble into isoform specific fibrous meshworks. We probe how the lamin meshworks influence the distribution of NPCs using 3D structured illumination microscopy (3D-SIM) with sub-pixel computational image analysis. We quantitatively describe how the distribution of NPCs is defined by the lamin meshwork fibers in wild-type mouse embryonic fibroblasts (MEFs), lamin isoform knockouts, and nucleoporin knockdowns. For each case, we determine the distance between NPCs and the lamin fibers by using adaptive resolution multi-orientation image analysis to localize the center of each structure on a subpixel basis with 10 nm precision. The results demonstrate that the centers of a majority of NPCs lie between 60 and 100 nm from lamin A, B1, and B2 fibers, a distance slightly greater than the NPC radius.

This analysis reveals that NPCs are laterally associated with lamins A, B1, and B2. In contrast, this spatial relationship is not detected for LC fibers. Relative to LC fibers, NPCs appear to be randomly positioned according to a uniform distribution. Taking advantage of the enlarged meshworks of lamin fibers in lamin A/C (LA/C) and B1 (LB1) knockouts, we show that NPCs follow the lamin fibers forming the perimeter of lamin depleted holes in LB1 and LA meshworks, respectively. When Nup153 and Elys are knocked down, NPCs are shifted away from lamin fibers comprising the sparse meshworks in lamin knockouts and fill gaps created in dense wild-type lamin meshworks. These results suggest that LA, LB1, and B2 act through specific nucleoporins to regulate the normal distribution of NPCs. Cryo-EM tomography combined with immunogold labeling reveals that there are direct physical interactions between lamin fibers and the nucleoplasmic ring of the NPC. Our quantitative approach combining 3D-SIM, cryo-electron tomography and computational image analysis sheds light on how lamins and nucleoporins interact and how the NL is organized. These interactions are pre-requisite to understanding a wide range of diseases, including the laminopathies caused by mutations in lamin A. Supported by NIH grants RO1GM106023,T32CA080621, and R35GM119619.

P1260/B389

Examining the Modular Architecture of the Nuclear Pore Complex through Targeted Degradation.

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The nuclear pore complex (NPC) is a channel through the nuclear envelope that allows macromolecular transport between the cytoplasm and nucleus. Disrupted NPCs and nucleocytoplasmic transport are hallmarks of many neurodegenerative diseases, including Huntington's and amyotrophic lateral sclerosis. NPCs contain roughly 30 proteins called Nups; Nup mutations are found in many tumors, further underscoring the need to better understand both individual Nups and NPC function overall. 32 copies of the vertebrate nonameric Y-complex assemble into rings that gird the inner and outer faces of the NPC. These rings are assumed to act as scaffolds of NPC architecture. To understand the roles of individual Y-complex subunits, we used CRISPR/Cas9 to add Auxin-Induced Degron (AID) tags at the genomic loci encoding Nup160, Nup133, Nup107, Nup96 and Nup85 in human DLD-1 cells. The tagged Nups assemble into functional NPCs and degrade rapidly (<4 hours) after auxin addition. In the resultant cell lines, we assessed how each Nup contributes to NPC structure and function. After Nup160, Nup133 or Nup85 depletion, NPCs retained non-tagged Y-complex members and stayed surprisingly intact and functional for transport. At the same time, post-mitotic NPC assembly was disrupted in their absence, suggesting that they are required for NPC formation but can be removed from intact NPCs without catastrophic consequences. By contrast, Nup96 or Nup107 depletion caused rapid dispersion of non-tagged Y-complex subunits and other Nups, particularly those from the cytoplasmic face of the NPC. Loss of Nup96 or Nup107 was also associated with substantially slower nuclear import. There results indicate that Nup96 and Nup107 are essential for maintenance of Y-complex ring structures and functional NPCs. Interestingly, mass spectrometry of nuclear envelope fractions from cells depleted Nup96 or Nup107 showed that not all Nups are dispersed upon their loss, and that members of the inner ring complex (IRC) were largely retained. To examine the relationship between the Y-complex and these retained Nups, we tagged Nup188, a member of the IRC. Depletion of Nup188 resulted in a pattern that inverse to the patterns generated after Nup96 or Nup107 loss, showing retention of the Y-complex but loss of most IRC components. These findings lead us to propose a model in which the NPC

has a modular architecture with interwoven pieces that can nevertheless stand in relative isolation from each other.

P1261/B390

Proximity Labeling for the Identification of *Plasmodium Berghei* Nucleoporins.

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The nuclear pore complex (NPC) is the gatekeeper for nucleocytoplasmic transport in all eukaryotic cells and plays a major role in the regulation of gene expression. Typically made up of 30 different proteins called nucleoporins (NUPs), the NPC forms a basket like structure interacting with, and controlling the transport of molecules in and out of the nucleus. Nucleoporins are widely conserved across eukaryotes but have diverged evolutionarily in the malaria parasite Plasmodium and are therefore difficult to identify. The protein composition of the Plasmodium NPC has remained largely elusive with the exception of five FG-repeat NUPs that likely line the central channel of the NPC. Using in vivo biotin proximity labeling (BioID) of FG-repeat NUPs in combination with mass-spectrometry we are exploring the NPC composition in the rodent malaria model Plasmodium berghei. To this end we are expressing BioID fusion proteins of the five P. berghei FG-repeat NUPs. Extraction and affinity-purification of the biotinylated proteins using a streptavidin column followed by mass spectrometry defined a proteomics data set of putative nuclear pore proteins including transmembrane domain NUPs. Immunofluorescence assays as well as labelling of BioID expressing parasites with streptavidin-fluorophore conjugate confirmed unique, parasite-specific proteins that localize to the nuclear periphery. We are currently exploring various in vivo proximity labeling techniques in the characterization of the Plasmodium NPC.

P1262/B391

Cul3 Substrate Adaptor SPOP Regulates the Nuclear Pore Protein NupJ.

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Cell processes like growth and division are tightly regulated. One such mechanism of regulation is ubiquitination. Ubiquitination can change a protein's localization or activity, or it can mark the protein for degradation by the ubiquitin proteasome system. The final step of ubiquitination, transferring ubiquitin to the target protein, is mediated by E3 ligases and their substrate adaptors, proteins that allow E3 ligases to be selective in choosing their targets. Understanding the targets of E3 ligases and substrate adaptors, then, is crucial to understanding cell regulation and disease mechanisms linked to misregulation of protein levels and activity. SPOP is a Cul3 E3 ligase substrate adaptor whose targets, such as c-Myc, PD-L1, and ERG, are crucial for cell cycle progression and cancer proliferation. Through a mass spectrometry screen, we identified SPOP as a potential regulator of NupJ, a nuclear pore protein. Knockdown of SPOP via siRNA in HeLa cells leads to increased protein levels of NupJ via immunoblotting, and SPOP and NupJ both co-localize at the nuclear envelope via immunofluorescence microscopy. Moreover, co-immunoprecipitation assays demonstrate that SPOP and NupJ bind to each other in *vitro*. Similar to overexpression of NupJ, siRNA against SPOP leads to an increase in the number of nuclear envelope defects. Overexpressed NupJ leads to defects in cell division. Our results suggest that SPOP targets NupJ for ubiquitin-mediated proteasomal degradation.

P1263/B392

Mps1 Releases Mad1 from Nuclear Pores to Ensure a Robust Mitotic Checkpoint and Accurate Chromosome Segregation.

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The Spindle Assembly Checkpoint (SAC) protects eukaryotic cells against chromosome mis-segregation by restraining the transition to anaphase in the presence of unattached kinetochores. This feedback mechanism works as a rheostat in which the signaling strength is defined by the amount of Mad1-Mad2 heterotetramers at kinetochores. During interphase Mad1 and Mad2 are associated with nuclear pore complexes through the nucleoporin Megator/Tpr. This arrangement is proposed to stabilize Mad1 and Mad2 and provide a scaffold for the assembly of pre-mitotic MCC. However, the molecular underpinnings controlling the spatiotemporal redistribution of Mad1-C-Mad2 as cells progress into mitosis remain elusive. Here, we show that Mps1-mediated phosphorylation of Megator/Tpr abolishes its interaction with Mad1 in *vitro* and in *Drosophila* cells. Timely activation of Mps1 during prophase triggers Mad1 release from nuclear pore complexes, which we find to be required for competent kinetochore recruitment of Mad1-C-Mad2 and robust checkpoint response. Importantly, preventing Mad1 binding to Megator/Tpr rescues the fidelity of chromosome segregation and aneuploidy in larval neuroblasts of *Drosophila mps1*-null mutants. Our findings demonstrate that the subcellular localization of Mad1 is stringently coordinated with cell cycle progression by kinetochore-extrinsic activity of Mps1. This ensures that both nuclear pore complexes in interphase and kinetochores in mitosis can generate anaphase inhibitors to efficiently preserve genomic stability.

P1264/B393

3d Genome Conformation in the Proximity of Nuclear Pore Defines HIV-1 Integration.

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Marina Lusic 3D genome conformation in the proximity of NPCs defines lentiviral integration Bojana Lucic[1], Maja Kuzman[2], de Castro IJ[1], Heinze Julia[1], Margiotta Erica[3], Beck Martin[3,4 and] Lusic Marina[1] *1 Heidelberg University Hospital, Heidelberg, 69120, Germany, German Center for Infection Research, Heidelberg, 69120, Germany 2. Bioinformatics Group, Division of Molecular Biology, Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia. 3. Structural and Computational Biology Unit, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany 4. Max Planck Institute of Biophysics, Frankfurt am Main* Integration into the cellular genome is one of the highlights of the lentiviral life cycle. The viral crosses the permeable barrier of the nuclear envelope (NE) and the Pre-integration complex (PIC), containing a reverse-transcribed genome, viral protein capsid (CA) and integrase (IN), docks to and is transported through nuclear pore complexes (NPCs). Our mechanistic understanding of these steps of the HIV life cycle is incomplete, although they are of utmost importance: by becoming an integral part of the host cell genome, the virus in disguise cannot be targeted by current antiviral treatments. Genes which are recurrently targeted by HIV-1, so called RIGs, position in the outer shells of the nucleus, are mostly active genes positioned in the outer shells of the

nucleus but excluded from lamina associated domains (LADs). These genes, as revealed by RNA Seq and ChiP Seq profiles are enriched in H3K27ac, H3K4me1 and BRD4, markers of super enhancers that drive cell-type-specific gene expression and are crucial to cell identity. By exploring the spatial gene positioning in activated CD4+ T cells, we found that genes with super-enhancers reposition towards the outer shells of the nucleus during T cell activation. This occurs during T cell activation, concomitant with the transcriptional upregulation of these genes and proved to be the sole property of genes with super-enhancers, and not of all active genes. Positioning of genes with Super-enhancers towards nuclear periphery prompted us to further explore the possibility that these genes belong to the NPC compartment. While the CHiP seq data using several Nups as anchoring points generated very poor profiles, the BiO ID profiles of gene of interest showed multiple interactions of Nup157 and Tpr (inner nuclear pore) whith proteins informed up.

P1265/B394

Nucleoporins Facilitate Heterochromatic Break Repair Off-pore.

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Heterochromatin is comprised of transponsons and large arrays of repetitive satellites prone to aberrant recombination. In *Drosophila*, homologous recombination is used to safely repair heterochromatic double-strand breaks (DSBs), however ‘safe’ completion of this process requires the break site to relocalize to the nuclear periphery before strand invasion and repair. Relocalization is dependent upon a transient actin/myosin network that pulls DSBs to the nuclear pore and inner nuclear membrane proteins (INMPs) where anchoring occurs in a Nup107 complex-dependent manner. We recently discovered an additional nucleoplasmic function of a subset of nucleoporins to facilitate relocalization of repair sites. Nup88, Nup98, and Sec13 are recruited to heterochromatic DSBs before relocalization in a SUMOylation-dependent manner, and are necessary for DSB mobilization (and not for anchoring to the nuclear periphery). However, nucleoplasmic nucleoporins are not necessary for actin filament formation, suggesting an actin-independent pathway for break mobilization. Depletion of these nucleoporins to the nuclear membrane results in heterochromatin repair defects and genomic instability. Together, these data reveal that Nup88, Nup98, and Sec13 function off-pore to promote heterochromatic DSB relocalization downstream from Smc5/6.

P1266/B395

The Role of Controlling Signals and Their Guidance of Werner's Helicase Interacting Protein 1 in Pathways Maintaining Genomic Stability.

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Werner's Helicase Interacting Protein 1 (WHIP or WRNIP1) has been shown to activate select molecular components of the ATM signaling pathway to maintain genomic stability and prevent instabilities from arising in subsequent cell cycles. In yeast, WHIP’s homologue MGS1 (maintenance of genome stability 1) knockouts exhibit an increased frequency in genetic errors, potentially leading to accelerated aging. Important to WHIP’s function is its localization in the nucleus, specifically at nuclear pore complexes. In our study, we utilize immunoprecipitation, deletion analysis, and immunofluorescence microscopy to demonstrate that WHIP’s localization is controlled by its predicted internal nuclear localization signal (NLS). Despite posttranslational modifications of WHIP, this signal is not controlled by phosphorylation

of neighboring serine threonine residues. As an extension of our investigations, we examined the localization of WHIP during mitosis. Our findings track the location of WHIP, as well as its co-localization with ATM signaling and mitotic components throughout the cell cycle. Thus far, our data suggests the cellular location of WHIP is important in sensing replication stress throughout the cell cycle.

5

Nuclear Bodies and Dynamics

P1267/B396

KDM2A-SF: a Demethylase Lacking the Demethylase Domain.

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The KDM2A histone lysine demethylase is present in mammalian cells as various isoforms. The canonical full-length KDM2A protein demethylates lysines of both histone and non-histone proteins. An alternative KDM2A mRNA isoform originates in *KDM2A* intron 12 and encodes a shorter protein that lacks the N-terminal Jumonji C demethylase domain. This short isoform, KDM2A-SF, contains all the other functional domain such as the CXXC DNA binding domain, PHD zinc finger domain, F-box domain and LRR domain. KDM2A-SF is thus able to bind to DNA, but it is unable demethylate lysines. We performed a detailed characterization of the *KDM2A-SF* mRNA using 5'RACE, northern blot and quantitative real time PCR. Although KDM2A-SF lacks the demethylase domain, it is, similarly to the long KDM2A isoform (KDM2A-LF), also ubiquitously expressed in various healthy and cancer cells. Further, our in *vitro* translation experiments showed that KDM2A-SF is translated from the start codon in the exon corresponding to exon 14 of the *KDM2A* full length mRNA. While we also explored nanotechnologies, immunofluorescence analyses of our KDM2A isoform specific knockdown experiments revealed that, unlike KDM2A-LF, KDM2A-SF forms distinct loci in the nucleus on pericentromeric heterochromatin in an HP1a-dependent manner. However, the functional consequences of this finding remain to be elucidated.

P1268/B397

Nuclear Architecture Modulates Transcriptional Dynamics.

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To facilitate effective gene regulation, the Human Genome is spatially organized into compartments, topologically associated domains (TADs), and loops. Transcription is governed by multiple Transcription factors (TFs) binding rapidly to genomic elements (enhancers and promoters) to promote gene activation. The coordinated role of compartments, TADs, and loops in transcriptional regulation is poorly understood. We show that real time Transcription Dynamics are altered by 3D nuclear organization. Specifically, ChIP-seq experiments and Single Molecule Tracking (SMT) of TFs in live cells demonstrate how cohesin and nuclear architecture regulate the interaction of Transcription Factors with chromatin in live human cells. To investigate the role of 3D genome structures in TF/chromatin interactions, we used computational methods to predict TAD borders, using CTCF binding data (ChIP-seq) and CTCF motif orientation. Interestingly, hormone-inducible TFs such as the Glucocorticoid Receptor (GR) bind

primarily within a TAD structure. Furthermore, we observed GR (and other TFs) bind principally to one of the TAD borders in an asymmetric manner, suggesting an expected role for TFs in the loop extrusion model. These preferred locations are co-bound by cohesin proteins (SMC1/3). Furthermore, loading of the Cohesin complex is potentiated upon activation of the glucocorticoid receptor, suggesting a possible synergy between GR and structural proteins to activate transcription. To explore the effect of Chromatin Loops on the Dynamics of Transcription Factors: we utilize the degron system (Auxin-RAD21mAID) for the acute depletion of RAD21, a core component of the Cohesin Complex. This produces an acute loss of chromatin loops. After rapid RAD21 depletion, we characterize the mobility of TFs using single molecule tracking (SMT). These factors include a hormone-inducible Transcription Factor, GR, and the architectural nuclear TF YY1, a fundamental interactor of the cohesin complex at enhancers. Acute depletion of RAD21 strongly reduces the binding of YY1 and GR to chromatin. Surprisingly, Chip-seq, ATAC-seq and RNA-seq show that, after RAD21 depletion, loss of TFs binding occurs exclusively at intergenic locations, while promoters are still bound by TFs such as YY1. Furthermore, the active H3K27ac mark is significantly altered after RAD21 depletion, suggesting a profound transition in the chromatin environment after rapid depletion of the cohesin complex. Our data suggest that nuclear structures are intimately involved in the real time interactions of Transcription Factors with chromatin targets.

P1269/B398

Mitogen-activated Protein Kinase P38 Mediates Heat Stress-induced Changes in Nucleolus-associated Chromatin Domains.

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Febrile conditions are evolutionary conserved components of innate immune responses that are beneficial for the survival of the organism. However,unchecked fever causes serious side effects. Stem cells are among the most vulnerable cell types, and damage to such cells can imperil organogenesis. Heat stress causes well-characterized changes in transcription within euchromatin. However, the impact of febrile conditions on heterochromatin organization of differentiated and embryonic stem cells remains elusive. Here, we have tested heat stress for effects on three-dimensional heterochromatin organization in human (IMR90) and mouse (MEFs) fibroblasts and human embryonic stem cells (H1). Specifically, we analyzed the association of Nucleolus-Associated Domains (NADs) with nucleoli via 3D DNA-FISH assays. Our initial observations suggest that distinct classes of NADs display differential sensitivity to heat stress. Specifically, “Type II” NADs that don’t also localize to the nuclear lamina appear more heat sensitive that “Type I” NADs that are frequently lamina-associated. In addition, via pharmacological inhibition, we observed that the activity of stress-activated MAPK p38 is required for NAD relocalization after exposure to elevated temperatures. These data illustrate how signal transduction machinery can reorganize the genome.

P1270/B399

Probing Macromolecular Crowding in the Cell Nucleus.

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The cell is filled with macromolecular complexes that must navigate through a crowded environment to perform their functions. Though macromolecular crowding is intrinsic to all cells, study of this physical property in the cell nucleus remains overlooked. Here, we are developing the use of genetically-encoded

multimeric nanoparticles (GEMs) (Delarue M et al. Cell [2018]) to probe macromolecular crowding in the nucleus and its effects on cell function using fission yeast. GEMs are spherical protein structures, ranging from 20 to 50 nm in diameter, that are labeled with fluorescent proteins. An alysis of the diffusive-like motion of these particles in the cell provides a measurement of local crowding within subcellular compartments. Further, we have developed methods to perturb macromolecular crowding. For example, we find that the addition of sorbitol, an osmotic agent, effectively promotes cellular crowding and decreases GEMs diffusion in both the cytoplasm and nucleus. In addition, we demonstrate that inhibiting ribosome biogenesis increases GEMs diffusion in both the cytoplasm and the nucleus, indicating that ribosomes may be a major crowding agent for both compartments. Finally, we find that upon inducing DNA damage, GEMs diffusion increases in the nucleus, which may have implications for DNA repair efficiency. In future studies, we hope to identify crowding factors in the nucleus and determine the relationship between crowding and nuclear function.

P1271/B400

Crowding within the Nucleus: Torc1 Takes Charge.

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The properties of the cell interior are crucial for the organization and efficiency of biochemical reactions, but the physical nature of the cytoplasm, nucleus and other organelles remains poorly understood, particularly at the mesoscale (10 nm - 1 μm). One of the key and underappreciated property of the cell interior is the high degree of macromolecular crowding. The diffusive and interactive behavior of molecules are significantly altered in a highly crowded environment, which in turn has a major impact on different biological processes and reactions. Macromolecular crowding is particularly relevant in the nucleus where large fraction of the volume is occupied by chromatin and the nucleolus. To date it remains a mystery how crowding within the nucleus is mechanistically regulated and what are the physiological consequences of perturbations to nuclear crowding. We have recently developed the nuclear-targeted Genetically Encoded Multimeric (GEM) nanoparticles that greatly facilitate the biophysical characterization of the cell. Key biophysical parameters including the degree of macromolecular crowding can be inferred from the motion of these probes. Using nuclear GEMs we show that inhibition of the TORC1 complex by rapamycin treatment decreases crowding within the budding yeast (*Saccharomyces cerevisiae*) nucleus. Genetic analyses revealed that downregulating rDNA transcription, a process that is tightly controlled by TORC1, phenocopies the effect of rapamycin treatment. Furthermore, TORC1 inhibition also decreased the size of the nucleolus, thus increasing the effective volume of the nucleoplasm. Therefore, we propose that TORC1 signaling adjusts molecular crowding within the nucleus by regulating rDNA transcription and/or the size of the nucleolus.

P1272/B401

Polyubiquitin Chain-induced Rnf168 Phase Separation Drives Dsdna Damage Repair Compartment.

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DNA damage response involves the formation of transient compartments at the site of the damage that concentrates the necessary signaling factors and repair complexes. For ssDNA repair, this enrichment is driven by the phase separation of FUS nucleated by PAR, but a similar mechanism for dsDNA repair is still unknown. Here we report that RNF168, an E3-ubiquitin ligase that ubiquitinates histones, forms

condensates in *vivo* upon DNA damage in a ubiquitin-dependent manner. These condensates show liquid-like properties as they recover upon photobleaching and are sensitive to hexanediol. Recombinant RNF168 alone does not phase separate but readily demixes upon addition of linear polyubiquitin chains. The phase behavior of these proteins is dependent on the salt conditions and the valency of the polyubiquitin chains used. Further, we show that these RNF168-polyubiquitin droplets are immiscible with FUS droplets providing a mechanism that prevents cross-talk between the ssDNA and dsDNA repair at the site of complex DNA lesions.

P1273/B402

Oligomerization as a General Mechanism for Enhancing Liquid-liquid Phase Separation.

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Liquid-liquid phase separation (LLPS) of proteins has been an attractive field of study in recent years, as it has been shown to be involved in cellular processes such as transcription, stress responses, and metabolite production. Oligomerization has previously been shown in some individual protein systems to be essential for LLPS of those proteins. In this work, we propose that oligomerization may be a universal mechanism by which any protein system can enhance LLPS. We demonstrate that RNA-binding protein TDP-43 is capable of LLPS in *vitro* without the use of its C-terminal prion-like domain, in a manner dependent on the oligomerization of its N-terminal domain (NTD). We show that fusing TDP-43's NTD to other proteins also enables or enhances their ability to LLPS, and we show that replacing TDP-43's NTD with other oligomerization domains enhances LLPS proportionately to the state of oligomerization. Finally, we demonstrate that oligomerization is used by TDP-43 to LLPS in *vivo*, where removing oligomerization abrogates LLPS. We believe oligomerization is a general mechanism to enhance LLPS, and that it will likely be seen as an important part of phase-separating systems in the future.

P1274/B403

The Nucleolus Is Composed of Two Distinct Types of Phase Separations.

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The nucleolus is encapsulated within the nucleus via liquid-liquid phase separation (LLPS). Recent studies suggest the high density of ribosomal DNA (rDNA) loops within the nucleolus is sufficient to generate a polymer-polymer phase separation (PPPS). How the polymer driven rDNA phase versus the RNA/protein liquid phase contribute to the integrity of the nucleolus is not well understood. We utilized live-cell fluorescent imaging of the rDNA locus and the ribonucleoprotein complex (CBF5) to distinguish the liquid from the polymer phases within the nucleolus of the budding yeast *S. cerevisiae*. In polymer phases DNA crosslinkers colocalize with DNA, and the density and binding kinetics of crosslinkers dictate the polymeric properties. In the liquid phase the components appear homogenous and surround the DNA. We found that condensin, a known DNA loop extrusion complex, and CDC14-GFP, a protein phosphatase sequestered in the nucleolus until the exit from mitosis, co-localize with the rDNA locus, indicative of a PPPS. In contrast, CBF5-RFP did not localize with the rDNA locus and instead appears homogenous surrounding and intermingled with the DNA. These data indicate that nucleolar ribonucleoproteins are part of a LLPS while rDNA is part of a PPPS. To determine if we could preferentially compact one phase from the other, we expressed a tetrameric LacI-GFP protein, which

can form DNA loops in a strain containing a LacO sequence integrated into all copies of the rDNA locus. The rDNA/LacI-GFP tetramer signal was significantly smaller than a rDNA/LacI-GFP dimer signal. However, the CBF5-mCherry signal area remained the same regardless of the LacI construct (dimer or tetramer). To test the role of rRNA in liquid phase formation we imaged CBF5-mCherry in cells containing 2-micron plasmids with a rDNA repeat under the expression of a galactose promoter. In these cells, the endogenous rDNA locus was removed, and a single rDNA repeat was placed on chromosome V. Multiple distinct CBF5 signals were observed throughout the nucleus in G1 and mitotic cells; however, upon transcriptional repression CBF5 appears as single signal. Thus, the liquid-like CBF5 phase can distribute throughout the nucleus in the absence of the rDNA polymer phase. The nucleolus is a composite structure with both polymer and liquid phase characteristics.

P1275/B404

Spatial Organization of Nucleoli in Macronuclei of Several Ciliate Species with Subchromosomal Organization of Macronuclear Genome.

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The nucleolus is an essential structural component of the nucleus. Three main structural subregions can be electron microscopically defined in nucleoli of higher eukaryotes: fibrillar centers (FC); the dense fibrillar component (DFC), partly or completely surrounding FC; and the granular component, located at nucleolar periphery. However, recent studies showed that such "classical" tripartite nucleolar organization is an evolutionary acquisition of higher eukaryotes. Ciliated protists contain two different types of nuclei in each cell: a germinal functionally inert micronucleus, and a somatic macronucleus, which is DNA-rich and transcriptionally active during vegetative growth. In contrast to genomes of metazoan cells, the macronuclear genomes are represented by sets of relatively short DNA molecules (minichromosomes) either of “gene size” (0.5-25 kb) or of subchromosomal size (from several tens up to several hundred kb). Thus, ciliate macronuclei are a convenient object to study the peculiarities of nucleolus morphology in the nuclei with minichromosomes. In this work, *Bursaria truncatella, Paramecium multinucleatum and Didinium nasutum* were studied. They belong to the species with subchromosomal organization of macronuclear genome. The aim of this work was to study nucleolar architecture in these species using confocal microscopy; 3D electron microscopy reconstruction on the basis of ultrathin sections; and localization of fibrillarin in the nucleoli using specific antibodies. Different spatial organization of nucleoli was observed. Fibrillarin is one of the highly evolutionary conserved nucleolar proteins, found in various species from *Archaea* to human. During interphase, fibrillarin is present in nucleoli of higher eukaryotes mainly in FC and DFC. It was shown that in *B. truncatella and P. micronucleatum* fibrillarin mainly occurs in the central region of the nucleoli, similar to nucleoli of higher eukaryotes. On the contrary, in the *D. nasutum* nucleoli fibrillarin was located at the periphery of nucleolar domain. These results are in a good agreement with the electron microscopic data on morphology of the nucleoli, which showed an unusual "inverted" location of the fibrillar component and the granular component in the nucleoli of *D. nasutum.* This work was supported by the Program of fundamental research for state academies for 2013-2020 years (01201363823).

P1276/B405

Ectopically Expressed Pno40 Suppresses Ribosomal Rna Synthesis by Inhibiting Ubf-dependent Transcription Activation.

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Ribosomal RNA (rRNA) production occurs in the nucleolus and is a critical process for ribosome biogenesis which affects protein synthesis capacity and determines the cell growth. Dysregulation of nucleolar homeostasis elicits a nucleolar stress response and is related to disease etiology, indicating that the regulation of nucleolar activity is crucial and tightly coordinated. We previously reported that nucleolar protein pNO40 overexpression mediates SR family splicing factors into the nucleolus and impairs mRNA metabolism, while the function of pNO40 in nucleolar homeostasis is unclear. Here, we demonstrate that overexpression of pNO40 downregulates RNA polymerase I transcription activity, resulting in pre-rRNA synthesis reduction and induces nucleolar segregation, a hallmark of rRNA synthesis inhibition and nucleolar stress response. Moreover, co-immunoprecipitation experiments revealed that ectopically expressed pNO40 interacts with UBF, a master transcription factor involved in pre-initiation complex (PIC) (containing SL-1 complex and RNA polymerase I complex) to activate and promote RNA polymerase I-mediated transcription, but disturbs its rDNA promoter binding ability. Collectively, our results demonstrate the role of pNO40 in rRNA biosynthesis regulation by compromising UBF function in rDNA transcription activation with subsequent rRNA synthesis inhibition.

P1277/B406

Sas10 Controls Ribosome Biogenesis by Stabilizing Mpp10 and Delivering the Mpp10-imp3-imp4 Complex to Nucleolus.

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Mpp10 forms a complex with Imp3 and Imp4 that serves as a core component of the ribosome small subunit (SSU) processome. Mpp10 also interacts with the nucleolar protein Sas10/Utp3. However, it remains unknown how the Mpp10-Imp3-Imp4 complex is delivered to the nucleolus and what biological function the Mpp10-Sas10 complex plays. Here we report that zebrafish Mpp10 and Sas10 are conserved nucleolar proteins essential for the development of digestive organs. Mpp10, but not Sas10/Utp3, is a target of the nucleolar-localized Def-Capn3 protein degradation pathway. Sas10 protects Mpp10 from Capn3-mediated cleavage by masking the Capn3-recognintion site in Mpp10. Def interacts with Sas10 to form the Def-Sas10-Mpp10 complex to facilitate the cleavage of Mpp10 by Capn3. Importantly, we find that Sas10 determines the nucleolar-localization of the Mpp10-Imp3-Imp4 complex. We conclude that Sas10 is essential not only for delivering the Mpp10-Imp3-Imp4 complex to the nucleolus for assembling the SSU processome but also for fine-tuning Mpp10 turnover in the nucleolus during organogenesis.

P1278/B407

Prostaglandins Restrict Nuclear Actin to Control the Nucleolus.

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Prostaglandins (PGs), lipid signals produced downstream of cyclooxygenase enzymes, have numerous physiological and pathological functions, including pain and inflammation, reproduction, heart health

and disease, and cancer development and progression. One means by which PGs act is through regulating the actin cytoskeleton. Actin is not solely cytoplasmic, but localizes and functions within the nucleus. Using the robust genetic system of Drosophila and the well-characterized developmental process of oogenesis, we discovered multiple pools of nuclear actin that exhibit distinct developmental and subnuclear localizations. Specifically, fluorescently conjugated DNase I labels monomeric actin in the nucleolus of every cell. Anti-actin C4 labels a monomeric, nucleolar actin in a subset of cells during early oogenesis and recognizes polymeric nuclear actin in both the oocyte and undifferentiated germline cells. Anti-actin AC15 labels polymeric nuclear actin that localizes to the chromatin starting at mid-oogenesis in both germline and somatic cells. Additionally, ectopic expression of GFP-Actin results in nuclear actin rod formation during early oogenesis. We find that PGs are required to limit the levels of the different pools of nuclear actin. Loss of the Drosophila cyclooxygenase enzyme, Pxt, results in increased GFP-Actin rod formation, an increased frequency of cells exhibiting C4 nuclear actin, and premature and increased levels of AC15 nuclear actin. Thus, PG signaling is a critical negative regulator of nuclear actin. Our findings that both DNase I and C4 label actin within the nucleolus, PGs regulate C4 nuclear actin, and PGs regulate the structure of the nucleolus, led us to hypothesize that PGs tightly control nuclear actin to regulate the nucleolus. Supporting this loss of Exportin 6, which mediates the nuclear export of actin, results in defects in nucleolar structure that are similar to those observed for loss of Pxt. These data suggest that nuclear actin plays a critical role in regulating nucleolar structure. Together our findings lead to the model that PGs tightly control nuclear actin to regulate nucleolar structure and likely function. As both the nucleolar localization and functions of actin and the role of PGs in regulating the nucleolus are conserved across organisms, our findings reveal a novel pathway that may play critical roles in both normal and pathological instances. Indeed, high levels of PGs, increased nuclear actin, and nucleolar changes are each independently associated with cancer development and progression.

P1279/B408

The Nucleolar Dense Fibrilar and Granular Components Observed by Atomic Force Microscopy.

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The nucleolus is a plurifunctional nuclear organelle involved in ribosome biogenesis and other functions. With the electron microscope three major ultrastructural components are distinguished, namely, dense fibrilar component, granular component and fibrilar centers. It is composed of rDNA, rRNA, pre-rRNA, UsnRNAs and some 700 proteins. While light and electron microscopy have revealed the major components, scarce data are available with the atomic force microscope. In addition, defining the differences among their component has been difficult using bi-dimensional approaches. Here, we use the atomic force microscope working in contact mode to study the components of the nucleolus of the tree *Ginkgo biloba*, adding the vertical resolution as a parameter, in semithin sections of material prepared as fir transmission electron microscope. The microscope can distinguished dense fibrilar component from granular component because they display differences when using profiles through the sections. These profiles are different from those of compact chromatin and nucleoplasm. The resolution given by the z resolution can be used as a parameter to distinguish both components one from each other. The present results suggest that dense fibrilar component may be distinguished from fibrilar centers by atomic force microscopy in other species as *Giardia lamblia*, where both components are intermingled (we thank CONACyT 180835, DGAPA-UNAM IN217917, PAPIME PE213916).

Endocytosis 2

P1280/B410

Upregulated Flotillins, New Markers of Metastatic Development, Deregulate Endocytosis and Vesicular Trafficking to Induce Epithelio-to-mesenchymal Transition and Cellular Invasion.

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Metastasis formation is the major cause of death in patient with cancer. Flotillin upregulation is detected in many invasive tumors, both carcinoma and sarcoma, and is a marker of poor prognosis associated with a higher metastatic risk. *How flotillins participate in the acquisition of invasive and metastatic properties remains to be determined. Through* in *vitro* and in *vivo* approaches, we showed that in non tumoral epithelial and mesenchymal cells, upregulation of flotillins is sufficient to induce epithelio-to-mesenchymal transition (EMT), extracellular matrix degradation and cellular invasion. Conversely, flotillin knockdown in invasive cancer cells reduces extracellular matrix degradation and cellular invasion. This illustrates the critical role of flotillin upregulation in the acquisition of invasive properties by cancer cells. Flotillin 1 and 2 are two ubiquitous, highly conserved and related proteins present in many cellular membrane compartments. They exist as hetero-tetramers that assemble in large oligomers to form molecular membrane scaffolds known to participate in membrane proteins clustering. When overexpressed, they induce the formation of plasma membrane invaginations and of intracellular vesicles and modify the trafficking of several cargos; promoting the Upregulated Flotillin-Induced Trafficking (UFIT) pathway. The cellular distribution of upregulated flotillins is dramatically modified with a strong enrichment in vesicular compartments that we characterized as non degradative-endolysosomes. *We aim at identifying how the UFIT pathway modifies the trafficking of key cargos leading to the acquisition of invasive properties. Through* various cutting-edge cell biology approaches (such as optogenetic to promote flotillin the UFIT pathway) and using global comparative analyses (transcriptomic, proteomic, phosphokinase arrays), we identified protein cargos of the UFIT pathway, such as key transmembrane signaling receptors, adhesion proteins and also the metalloproteinase MT1-MMP, whose endocytosis, trafficking and activity is modified by flotillin upregulation. We propose that the UFIT pathway generates flotillin-positive endolysosomes acting as a “signalosome and recycling compartment” involved in the activation of oncogenic signaling pathways stimulating EMT and cellular invasion.

P1281/B411

Functional an alysis of Mitochondria-endosome Interaction in the Regulation of Endocytosis.

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Endocytosis mediates the internalization and ingestion of a variety of endogenous or exogenous substances, including receptors, nutrients, and pathogens, into cells under the control of intracellular signaling. We have previously reported that the complex formed by the small GTPase Ras and phosphoinositide 3-kinase (PI3K) is translocated from the plasma membrane to endosomes upon epidermal growth factor (EGF) stimulation, and Ras-PI3K signaling thereby promotes endocytosis and endosome maturation (***Cell Signal***21: 1672, 2009; ***PLoS ONE***6: e16324, 2011). We have also reported

that endosomal localization of the Ras-PI3K complex is regulated through N-terminus region of the Ras-binding domain of PI3K, which is named RAPEL after Ras-PI3K endosomal localization (*Cell Struct Funct* 44: 61, 2019). To reveal the mechanism underlying these phenomena, we performed yeast two-hybrid assay to screen the binding proteins of RAPEL and identified six candidate proteins. Among these candidates, we focused on voltage-dependent anion channel 2 (VDAC2), a mitochondrial outer membrane protein that permeates ions and small molecules through its pore. When cells in which VDAC2 was knocked down were subjected to time-lapse microscopy, it was revealed that the endosomal localization of the Ras-PI3K complex was suppressed. In addition, uptake of dextran, which is a known substrate for endocytosis, was reduced and increased by the knockdown and overexpression of VDAC2, respectively. These data indicated that VDAC2 is involved in the spatiotemporal regulation of Ras-PI3K signaling and subsequent promotion of endocytosis. Next, we observed the intracellular dynamics of mitochondria and endosomes, and found that they transiently interacted with each other. Moreover, the mitochondria-endosome interaction was promoted by EGF stimulation, and such promotion was suppressed by knockdown of VDAC2. Therefore, to investigate the role of mitochondria-endosome interaction in the regulation of endocytosis, we developed an optogenetic approach, using cryptochrome 2 and cryptochrome 2-interacting basic helix-loop helix 1, so that the interaction is brought about by blue-light illumination. Interestingly, when the interaction was induced by light, endosome acidification was facilitated in a VDAC2-dependent manner. In conclusion, VDAC2 promotes endosomal localization of the Ras-PI3K complex and facilitates endosome maturation through mitochondria-endosome interaction.

P1282/B412

Feedback Effect of GGA3 Phosphorylation on TrkA Endocytic Trafficking.

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TrkA post-endocytic sorting significantly influences neuronal cell survival and differentiation. The process that directs the sorting of TrkA to diverse pathways have a significant impact on TrkA-mediated biological functions. We have demonstrated that GGA3 (Golgi-localized γ adaptin-ear-containing ADP ribosylation factor-binding protein 3) interacts directly with the TrkA cytoplasmic tail through an internal DXXLL motif and mediates the functional recycling of TrkA to the plasma membrane. We identified GGA3 as a key player in a novel DXXLL-mediated endosomal sorting machinery that targets TrkA to the plasma membrane, where it prolongs the activation of Akt signaling and survival responses. Indeed, GGA3 has been shown to be crucial for the sorting of EGFR and BACE to lysosomes through interactions with ubiquitin and for the recycling of the TrkA and Met receptor to the PM through an interaction with the DXXLL motif. Given that TrkA is ubiquitinated following ligand binding, we wonder how GGA3 preferentially uses the DXXLL motif to target TrkA to the recycling pathway. The phosphorylation of GGAs themselves has been shown to be a key factor in the regulation of their function. It would be appropriate to examine whether NGF stimulation leads to the phosphorylation of GGA3 and changes its binding capacity to ubiquitin or DXXLL motif, controlling the sorting fate of endocytic TrkA. Therefore, we aimed to check the phosphorylation status of GGA3 protein upon NGF stimulation and the feedback effects on TrkA endocytic sorting fate decision. We found NGF treatment could induce a molecular weight increase of GGA3 in PC12-615 cells, probably a result of phosphorylation. Ongoing studies are investigating the exact phosphorylated residues of GGA3 induced by NGF, and then we will make clear the consequences of the phosphorlation on GGA3 function, as well as on TrkA endocytic trafficking and physical functions.

P1283/B413

Cadherin Is a Cargo of WAVE-dependent Trafficking in Mature Epithelia.

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We are interested in how WAVE and Cadherin function together to maintain stable and healthy Apical Junctions (AJs) in mature epithelia. WAVE is a branched actin nucleation promoting factor and Cadherin is a transmembrane cell adhesion protein. Both WAVE and Cadherin regulate cell polarity and are also misregulated in many cancers. Our previous work about establishment of apical junctions in developing *C. elegans* embryos revealed interdependence between the WAVE and Cadherin complexes. They become enriched at similar times in development, and loss of WAVE or Cadherin suppresses F-actin enrichment at the AJs of epithelia. These results agree with findings in tissue culture which implicate WAVE in promoting formation of the Cadherin junction. We became intrigued by the effects of WAVE and branched actin on Cadherin enrichment and dynamics at the AJ. Interestingly, some regulators of endosomal recycling had similar effects on Cadherin. Therefore, in this presentation we focus on the unexplored role of WAVE at the endocytic recycling compartment (ERC). Cadherin is transported via ERC by RAB-10 and RAB-11 GTPases. Downregulation of WAVE caused imbalance of RAB-10 and RAB-11 vesicular transport at ERC. In the absence of WAVE, RAB-10 levels as well as vesicle number and velocity decreased. In contrast, loss of WAVE led to higher RAB-11 levels and vesicle velocity. RAB-10 depletion had different effect on RAB-11 dynamics suggesting that WAVE has distinct roles at RAB-10 and RAB-11 endosomes. Since vesicle formation, or movement, or both may be affected by WAVE-dependent branched actin, we are imaging and assessing actin presence and dynamics at ERC-associated vesicles. This is the first report elucidating the role of WAVE and branched actin in Cadherin trafficking and recycling at the ERC. Our research will explain how WAVE supports homeostatic accumulation of Cadherin at AJ in mature epithelia. Investigating WAVE-dependent Cadherin recycling may inform cancer treatments and may also reveal mechanisms of subversion of RAB-11-mediated delivery of Cadherin to AJs by various pathogens.

P1284/B414

Selective Uptake of Carboxylated Multi-walled Carbon Nanotubes Impairs Macrophage Phagocytic Activity Via Class a Type 1 Scavenger Receptors.

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The global production and applications of multi-walled carbon nanotubes (MWNTs) have increased despite evidence that MWNTs cause pulmonary fibrosis in lab animals that may lead to mesothelioma. Studies to understand the pathological mechanisms of MWNTs often focus on macrophages as they are first responders to invaders in the body. Recent work in our lab shows that macrophages preferentially accumulate ~100X more carboxylated MWNTs (C-MWNTs) than pristine MWNTs (P-MWNTs). Also, Class a scavenger receptors type 1 (SR-A1) expressed in macrophages may be involved in selective C-MWNTs uptake (Wang et. Al., *Nanotoxicology*, 2018). We are currently investigating the accumulation of C-MWNTs by macrophages via SR-A1 receptors and their effects on phagocytic function: **1. Selective C-MWNTs accumulation correlates with surface SR-A1 receptor expression.** Three mouse macrophages and two Chinese Hamster Ovary (CHO) cell lines were used to demonstrate the specificity of SR-A1 receptors for C-MWNTs uptake. Both RAW 264.7 and wild type B6 mouse alveolar macrophages express

high SR-A1 receptors with active C-MWNTs uptake whereas ZK cells (alveolar macrophages derived from a SR-A1 knockout mouse) deficient in SR-A1 receptors fail to accumulate C-MWNTs. Non-phagocytic cell types, such as epithelial CHO cells, express low SR-A1 receptors and support little MWNTs uptake. Mutant CHO clones that express transfected mouse SR-A1 receptor genes show increased uptake of C-MWNTs but not of P- or N-MWNTs. In addition, the surface SR-A1 receptor levels was reduced by 50% in RAW 264.7 cells after a 24h continuous exposure to C-MWNTs, suggesting that active C-MWNTs phagocytosis may deplete SR-A1 receptors from the cell surface. **2. C-MWNT accumulation impairs phagocytic function via SR-A1 receptors. To** assess the potential adverse effects of C-MWNT accumulation in macrophages, RAW 264.7 cells were pre-exposed to C-MWNTs for 24h at 37 °C, washed, and then tested for the subsequent phagocytosis of ligands that are known to interact with SR-A1 receptors. The uptake of three fluorescent-conjugated phagocytosis markers, including polystyrene beads, heat-killed E. coli, and oxidized LDL (oxLDL), were assessed using confocal fluorescence microscopy and measured quantitatively by flow cytometry. There were significant reductions in the uptake of polystyrene beads and E. coliin cells pre-treated with C-MWNTs but not in cells pre-treated with either P- or N-MWNTs, compared to the untreated control cells. The study on oxLDL uptake is underway. These results confirmed the selective uptake of C-MWNTs in macrophages via SR-A1 receptors. In addition, prolonged high C-MWNT uptake correlate inversely with subsequent SR-A1-dependent phagocytic activities in macrophages.

P1285/B415

Oligo-arginine-bearing Tandem Repeat Penetration-accelerating Sequence Delivers Protein to Cytosol.

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Cell-penetrating peptides (CPPs) can deliver cargo proteins into cells; however, internalized CPP-cargo is mainly retained in endosomes. To facilitate the cytosolic delivery of larger molecules such as proteins, we developed a new CPP sequence, named Pas2r12, consisting of a repeated Pas sequence (FFLIG-FFLIG) and D-dodeca-arginine (r12). This peptide significantly enhanced the cellular uptake and cytosolic release of green fluorescent protein and immunoglobulin G.However, the cytosolic EGFP deliverywas suppressed by caveolae-mediated endocytosis inhibitors. These results suggest that Pas2r12 enhances membrane penetration of cargo macromolecules without the need for crosslinking, and that caveolae-mediated endocytosis may be the route by which cytosolic delivery is enhanced. (Ref.) Okuda et al.: Biomacromolecules 2019 20 (5) 1849-1859

P1286/B416

Vps501, a Novel Vacuolar SNX-BAR Protein That Cooperates with the Sea Complex to Regulate Autophagy.

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The endosomal system facilitates the sorting and export of cargos that are packaged and conveyed by tubular and vesicular carriers destined for plasma membrane recycling or Golgi retrograde transport pathways, in part through the action of members of the sorting nexin (SNX) family of proteins. In particular, the SNX-BAR proteins, a sub-family of sorting nexins characterized by the presence of a C-terminus dimeric Bin/Amphiphysin/Rvs (BAR) lipid curvature domain, and the ability to recognize phosphatidylinositol 3-phosphate through an evolutionarily conserved Phox-Homology domain, are of

great interest. In budding yeast, the SNX-BAR subfamily has traditionally been thought to consist of six members, all of which are well-characterized. Sequence and phylogenetic analyses indicated the existence of a seventh member of this group. We now report the identification and functional characterization of this novel SNX-BAR, we have named Vps501. Using high-resolution fluorescence microscopy, we have found that Vps501 uniquely localizes to the vacuole and interacts with autophagy regulators, namely subunits of the SEA complex and TORC1. Furthermore, cells lacking Vps501 display a deficiency in starvation induced, nonselective autophagy that is severely exacerbated by ablation of subunits of the SEACIT. We also report that Vps501 interacts with the autophagy related protein Atg27 and regulates the dynamics of Atg27 trafficking at the vacuole. We hypothesize that Vps501 interacts with the SEA complex to negatively regulate autophagy via a novel vacuole membrane trafficking pathway. By contributing to a fuller understanding of the roles SNX proteins can play in cellular function, this discovery may inform future investigations of SNX-associated processes such as pathogen invasion or human conditions associated with defective SNX proteins, including neurodegenerative diseases and cancer.

P1287/B417

Analysis of Retromer Complex Dynamics on Supported Lipid Bilayers.

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In the endocytic system, vesicles from the plasma membrane containing lipids and integral membrane proteins fuse with sorting endosomes. There these cargoes are sorted and delivered back to the plasma membrane or to other organelles by the process of retrograde trafficking.The retromer complex, a trimeric (proteins Vps35, Vps26, and Vps29) endosomal sorting device , is a key component of retrograde trafficking and recognizes and sorts specific cargoes to multiple destinations. Despite the unequivocal importance of retromer in retrograde trafficking, the mechanism of how retromer sorts and packages cargo into retrograde sorting vesicles is unknown. It has been proposed that retromer sorting (i.e. cargo concentration/clustering) is facilitated first by intrinsic oligomerization of the retromer complex bound to cargo, which would begin to drive cargo into nascent sorting tubules. Further enrichment/concentration is proposed to be induced through an interaction with the WASH complex member Fam21, which has 21 retromer binding sites.We reconstituted the retromer coat complex assembly on a supported membrane bilayer, which allowed us to systematically probe the role of Fam21 and retromer interaction. In the reconstituted system we have both the membrane and the essential proteins of the retromer cargo-sorting complex, allowing us to investigate how these components interact and assemble on a supported bilayer using TIRF microscopy. When associated with the membrane, retromer exists as a distribution of oligomers, with a mean of eight subunits. Surprisingly, despite the presence of multiple retromer binding sites in Fam21, direct interaction between Fam21 and retromer on the bilayer does not influence the distribution of retromer oligomers. In addition, the oligomer distribution of retromer was not influenced by the presence of accessory coat factors that bind retromer, including Rab7-GTP, Sorting nexin 3 (Snx3), or cargo. These data suggest that, contrary to long-standing hypotheses, retromer does not substantially oligomerize on a membrane, either by itself, or in the presence of cargo, accessory factors, or the Fam21/WASH complex.

P1288/B418

COMMD1 and PtdIns(4,5)P_2 interaction maintain ATP7B copper transporter trafficking fidelity in HepG2 cells.

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Copper-responsive intracellular ATP7B trafficking is critical to maintain copper balance in mammalian hepatocytes and thus organismal copper levels. The COMMD1 protein binds both the ATP7B copper transporter and phosphatidylinositol (4,5)-*bis*phosphate (PtdIns(4,5)P_2), while COMMD1 loss causes hepatocyte copper accumulation. Although it is clear that COMMD1 is included in endocytic trafficking complexes, a direct function for COMMD1 in ATP7B trafficking has not been defined. In this study, experiments using quantitative fluorescence microscopy reveal that COMMD1 modulates the copper-responsive ATP7B trafficking through recruitment to PtdIns(4,5)P_2. Decreased COMMD1 abundance results in loss of ATP7B from lysosomes and the *trans*-Golgi network (TGN) in high copper conditions, while excess expression of COMMD1 also disrupts ATP7B trafficking and TGN structure. Overexpression of COMMD1 mutated to inhibit PtdIns(4,5)P_2 binding has little impact on ATP7B trafficking. A mechanistic PtdIns(4,5)P_2-mediated function for COMMD1 is proposed that is consistent with decreased cellular copper export due to disruption of the ATP7B trafficking itinerary and accumulation in the early endosome when COMMD1 is depleted. PtdIns(4,5)P_2 interaction with COMMD1 as well as COMMD1 abundance may both be important in maintenance of specific membrane protein trafficking pathways.

P1289/B419

Yeast Eps15 Homologue Pan1p and Epsin Homologues Ent1p/ Ent2p Cooperatively Function to Transport Clathrin-coated Vesicle Along Actin Cable.

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In yeast endocytosis, actin assembly is required for both internalization and transport of newly formed vesicles away from the plasma membrane. In the previous work, we have shown that phosphorylation of yeast Eps-15 like protein Pan1p induces disassembly of coat proteins, as well as actin filaments by blocking the interaction between Pan1p and actin filaments. Recently, we showed that dephosphorylated mutant of Pan1p (Pan1-18TA) causes abnormal cytoplasmic actin clumps, and persistent association between vesicles and actin cables. Interestingly, these interactions are partially suppressed in Pan1-18TAΔ855 mutants that lack its C-terminal actin binding and Arp2/3-activating regions. Thus, Pan1p seems to be one of the key regulators that fixe vesicles to the actin cable and then dissociate from the cable upon phosphorylation. However, the ability of vesicles to bind to actin cables in Pan1-18TAΔ855 was not completely lost in the mutant, implying the existence of additional actin-binding coat protein(s) that stabilize vesicle association with actin cables. To test whether Epsin homologue, Ent1p and Ent2p, participate in the interaction between vesicles and actin, we combined ent1*Δ* or ent2*Δ* with *Pan1-18TA Δ855* mutant. In these mutant cells, the number and size of actin containing clumps are significantly reduced, although substantial numbers of clumps containing Pan1p are still formed, suggesting actin binding ability of endocytic vesicle was largely disrupted in these mutant cells. To further examine the contribution of Pan1p, Ent1p and Ent2p in binding of endocytic vesicle with actin cable, we made single, double or triple mutants in which actin binding regions of these proteins were deleted. As a result, we observed significant defects in dynamics and transport of

endocytic vesicle in the *pan1 ent1 ent2* triple mutant cells. These results suggest that actin binding activities of these endocytic coat proteins is important for transport of endocytic vesicles along actin cable.

P1290/B420

Nutrient Regulation Is Potentially Compromised in Cells with Disrupted Endocytosis and ESCRT-Dependent Protein Sorting.

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Endocytosis is a vital cellular mechanism that involves the uptake of transmembrane proteins, referred to as cargo and extracellular material. Deletion of endocytic genes can lead to diseases such as neurodegenerative and lysosomal storage disorders. Specifically, clathrin-mediated endocytosis (CME) is a process in which a clathrin coat assists in membrane invagination. Endocytic adaptor proteins bind to transmembrane cargo and recruit clathrin. Previous findings indicated that cells lacking four adaptor proteins, the yeast epsins, Ent1 and Ent2, and AP180 homologues, Yap1801 and Yap1802, while maintaining a single epsin ENTH domain for viability (4Δ+ENTH), led to severely inhibited cargo internalization. We performed an extensive screen for synthetic lethality using a 4Δ+ENTH strain that expresses a full-length Ent1 cover plasmid. One set of synthetic lethal interactions with the 4Δ+ENTH background resulted from mutations in *VPS4*. Vps4 assists in the disassembly of Endosomal Sorting Complexes Required for Transport (ESCRT), which contribute to the degradation of endocytic cargo by trafficking transmembrane proteins from the endosomal membrane to endosomal lumen. Further experimental results indicated that inactivation of the large ATPase domain of Vps4 led to a phenotype similar to a 4Δ+ENTH *vps4*Δ strain, showing that this ATPase activity is essential for the viability of 4Δ+ENTH cells. To determine if mutations in other endocytosis genes exhibit negative genetic interactions with the loss of *VPS4*, we performed an experiment with cells lacking End3, a late coat CME protein. In previous experiments, an *end3*Δ *vps4*Δ strain also appeared to be synthetic lethal, in that a plasmid expressing full-length End3 was required for viability when the cells were grown on medium that selected against the End3 plasmid. However, data obtained using a different approach showed that the *end3*Δ *vps4*Δ cells constructed without an End3 plasmid were severely slow-growing but viable on rich medium. Due to this result, a 4Δ+ENTH *vps4*Δ strain was re- streaked on counterselective medium supplemented with sorbitol for osmotic support, which allowed the cells to grow without the full-length Ent1 plasmid. These 4Δ+ENTH *vps4*Δ cells were then viable on minimal medium without counterselection or sorbitol, but were not viable on rich medium. Together, our results indicate that cells with endocytic and ESCRT-mediated trafficking defects are synthetic sick instead of synthetic lethal and can be rescued by osmotic support. Also, these cells are able to proliferate at higher rates on minimal medium than rich medium, which suggests that nutrient regulation may be comprised in this genetic context.

P1291/B421

Effect of Ap2 µ2 Serine 45 Phosphorylation on Clathrin-mediated Endocytosis.

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Clathrin-mediated endocytosis (CME) is one of the pathways, by which eukaryotic cells internalize receptors and their ligands. The process starts at the plasma membrane and over 50 different proteins are involved in the formation of clathrin-coated pit (CCP), which mature and after scission become clathrin-coated vesicle (SSV). The assembly of CCP is initiated by the multisubunit adaptor complex 2 consisting of α, β2, µ2 and σ subunits. In cytoplasm AP2 exists in inactive- closed conformation. Binding to the plasma membrane triggers the conformational changes of AP2, which lead to open, active state and subsequent cargo binding and clathrin recruitment. Our initial results revealed that inhibition of mTOR/S6K1 pathway alters CME. Therefore the aim of the study was to understand a molecular mechanism underlying such regulation. We used immunoprecipitation, in vitro kinase assays and mass spectrometry to assess functional interaction between mTOR pathway and AP2 as well as identification of phosphorylation sites. Moreover formation and internalization of CCPs was performed with TIRF microscopy. This latter was studied using transfected or stable HeLa cell lines. Here we report that AP2 µ2 binds S6K1 and is phosphorylated on S45 in the S6K1 dependent manner. Substitution of serine 45 by alanine residue of µ2 inhibits transferrin uptake. Proximity ligation assay revealed decreased interaction of TrfR with phospho-deficient µ2. Quantitative live-cell analysis showed that in cells expressing µ2 S45A mutant CCPs initiation, lifetime distribution of CCPs and clathrin recruitment is altered. The results of our study have revealed importance of AP2 µ2 serine 45 phosphorylation for clathrin-mediated endocytosis. *This work has been supported by Polish National Science Centre grants* 2016/21/B/NZ3/03639 and 2017/25/N/NZ3/01280.

P1292/B422

Disassembly Asymmetry Score Classification: a Novel and Robust Tool for Phenotype Detection in Clathrin-mediated Endocytosis.

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Clathrin-mediated endocytosis (CME) is a resilient machinery for cargo internalization in mammalian cells that involves 70+ endocytic accessory proteins (EAP). Perturbation of single EAP usually causes minor/none effect in biochemical measurement of CME outcome, resulting in inconclusive/misleading information regarding in *situ* functions of these EAPs. Live-cell imaging cues potential earlier roles of EAPs prior to the finale of CME. However, for years, this approach is contaminated by abortive-clathrin coat (AC) that interferes measurement of the bona fide cargo carrier, clathrin-coated pit (CCP). Here, we develop a thermodynamics-inspired method, “disassembly asymmetry score classification (DASC)”, that unambiguously separates ACs from CCPs without additional marker. After packaging and extensive verification, we use DASC-identified ACs and CCPs to quantify the dynamics of CME progression in 10 EAP knockdown conditions. DASC detects diverse phenotypes in multiple conditions where biochemistry shows no functional consequence, suggesting this new tool a necessity for uncovering EAP functions in CME dynamics.

P1293/B423

Hiv-1 Nef Hijacks Clathrin-mediated Endocytosis (CME) to Deplete Anti-viral Factors.

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HIV-1 evades immune killing, and if left untreated, leads to acquired immune deficiency syndrome (AIDS). A cure has not been found largely due to the virus' ability to evade immune detection by removing important host defense factors from the cellular membrane through clathrin-mediated endocytosis (CME). The HIV-1 accessory protein, Nef, is the main culprit involved in hijacking CME by down regulating many host proteins to maximize viral survival and fusion. CME is one pathway in which cells internalize membrane-bound receptors. Cargo recognition is mediated by the CME adaptor protein-2 (AP2), through the YXXΦ and dileucine signal binding sites. AP2 is tightly regulated in cells. The signal-binding sites are not accessible in the 'locked' conformation. Nef induces the removal of these surface proteins by hijacking CME, potentially by activating AP2 and exposing the signal binding sites. However, the exact mechanism by which Nef induces down-modulation of surface proteins, many of which lack canonical AP2-interacting domains, is not known. Recent studies have identified SERine INCorporating protein 3 and 5 (SERINC3 and SERINC5) as potent antiviral factors that are down regulated in a Nef-dependent manner. Virions produced in cells with properly localized SERINCs have reduced infectious potency. Live-cell TIRF imaging shows that Nef and the SERINCs are recruited to clathrin-coated pits at approximately the same time as clathrin. Using an inducible Nef expression system, I showed that acute Nef expression causes a reduction of AP2 lifetime in Jurkat cells from an average of 40sec to 30sec. Using a combination of live-cell TIRF imaging and advanced quantitative analysis, I was able to determine the dynamics of Nef recruitment to CME sites, and how this recruitment affects AP2 and dynamin-2 lifetimes. These data may provide clues on the mechanism by which Nef modulates host CME to induce the removal of membrane-bound cellular defense proteins.

P1294/B424

Regulation of Membrane Scission in *S. Cerevisiae* by Bar Domain Proteins.

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In clathrin-mediated endocytosis, a flat plasma membrane is pulled into an invagination that eventually forms a vesicle. In mammalian cells, forces that drive the transition from invagination to spherical vesicle are provided by the GTPase Dynamin that acts in concert with crescent-shaped BAR domain proteins. In yeast cells, what causes membrane scission is unclear. The yeast BAR domain protein complex Rvs161/167 (Rvs) is nevertheless an important player: deletion of Rvs reduces scission efficiency by 30%. A mechanistic understanding of the influence of Rvs on scission however, remains incomplete. We found that arrival of Rvs to endocytic sites is timed by interaction of its BAR domain with specific membrane curvature. A second domain of Rvs167- the SH3 domain- affects localization efficiency of Rvs as well as dynamics of membrane invagination. This indicates that both BAR and SH3 domains are important for the role of Rvs as a regulator of scission. We tested current models of membrane scission, and found that deleting yeast synaptojanins or dynamin does not change scission dynamics. Interfacial forces caused by synaptojanin-mediated lipid hydrolysis are therefore unlikely to be sufficient for scission, and forces exerted by dynamin are not required. Invagination length is insensitive to overexpression of Rvs, suggesting that the recently proposed mechanism of BAR- induced protein friction on the membrane is not likely to drive scission in yeast. Contrary to the prediction of this model, deleting Rvs instead reduces invagination lengths at which scission occurs. We propose that

recruitment of Rvs BAR domains delays scission and allows invaginations to grow by stabilizing them. We also propose that vesicle formation is dependent on forces exerted by a different module of the endocytic pathway, the actin network. Preventing premature membrane scission via BAR interaction could allow invaginations to grow to a particular length and accumulate enough forces within the actin network to reliably cut the membrane.

P1295/B425

Ppp1r21, Core Component of the Novel Rab-5 Effector Complex, Is Essential for Early Zebrafish Morphogenesis Via Regulation of Cadherin Turnover and Wnt Signaling.

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The endocytic system has emerged as an essential integrative layer regulating signal transduction and cell-cell adhesion in a cell-context dependent manner. How cargo flux of key proteins through the endosomal network contributes to the integration of core embryonic processes during development remains largely unknown. We recently identified a novel Rab5 effector protein complex that regulates cargo trafficking at the early endosomal level in mammalian cell culture. PPP1R21 is a core subunit of this complex and has been recently implicated in a severe neurodevelopmental human disorder. Here, we report that PPP1R21 localizes to Rab5-positive early endosomes in vivo and is required to maintain cadherin-mediated cell adhesion integrity and WNT/β-catenin signaling activity during zebrafish gastrulation. Knockdown of zebrafish ppp1r21 does not disrupt the early endocytic system in vivo but modulates endosomal transport of E-cadherin and β-catenin, consequently altering their subcellular localization. ppp1r21 knockdown results in both reduced E-cadherin levels at the cell membrane and a reduction of β-catenin in the nuclei. That in turn correlates with reduced WNT/β-catenin signaling activity, progressive loss of cell-cell adhesion and collective cell migration defects in the whole embryo. Our data demonstrates that PPP1R21 is required for the precise regulation of endosomal transport of E-cadherin and β-catenin during zebrafish gastrulation and suggests that the functional integration of WNT/β-cateninsignaling with E-cadherin-mediated cell adhesion depends on endocytic regulation of limited protein pools. We propose that parallel/synergistic endosomal transport of proteins functions as a mechanism to integrate core processes such as cell adhesion and signaling during vertebrate development and explain how disruption of this process could lead to complex neurodevelopmental syndromes observed in humans.

Endosomes, Lysosomes, and Lysosome-Related Organelles 1

P1296/B426

Endosomal PI(3)P Regulation by the COMMD/CCDC22/CCDC933 (CCC) Complex Controls Membrane Protein Recycling.

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Proper membrane protein recycling through the endolysosomal system relies on a variety of protein complexes that interact with cargo proteins, phospholipid membranes, and effector molecules. The COMMD/CCDC22/CCDC93 (CCC) complex plays a critical role in recycling events by regulating the action of both Retromer and Retriever, two multiprotein complexes that function at endosomes to sort the

cargo proteins either to the Golgi apparatus or to the plasma membrane. However, the mechanism by which the CCC complex can impinge on both pathways remains unexplained. In this study, we show that the integrity of the CCC complex is required to maintain normal levels of phosphatidylinositol-3-phosphate (PI(3)P) within the endosomal compartment. Absence of this complex leads to elevated PI(3)P levels, which we show result in enhanced recruitment and activation of the WASH complex, an actin nucleation promoting factor. Furthermore, this leads to hyper-accumulation of endosomal F-actin and trapping of internalized membrane proteins. Mechanistically, we find that the CCC complex modulates PI(3)P levels by regulating the phosphorylation and endosomal recruitment of the lipid phosphatase MTMR2. Taken together, the CCC complex emerges as a key regulator of endosomal PI(3)P levels that is critical to endosomal cargo recycling pathways.

P1297/B427

Rin3 Binds to Bin1 and Cd2ap to Increase App-ctfs in Early Endosomes.

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Background: Alzheimer's Disease (AD) is one of the most common neurodegenerative disorders. Recent genome-wide association studies (GWAS) and meta-analysis have uncovered more than 20 genes as potential risk factors of AD. Among them, RIN3 (the Ras and Rab Interactor 3) is a guanine nucleotide exchange factor (GEF) for the small Rab5 GTPase family and has been implicated as a risk factor for both late onset AD (LOAD) and sporadic early onset AD (sEOAD). However, it remains unknown how expression of RIN3 is linked to AD pathogenesis. **Methods**: Quantitative PCR and immunoblotting were used to measure the RIN3 expression level in brain tissue and cultured basal forebrain cholinergic neurons (BFCNs) from both APP/PS1 transgenic mice and Wild Type (WT) controls. Immunostaining was used to visualize Rab5 positive endosome is cultured primary BFCNs from APP/PS1 mice and WT mice. Recombination RIN3-flag-tagged proteins were expressed and purified from HEK293T cell line. Mass Spectrometry was used to define proteins associated with RIN3. Co-IP, immunofluorescence and yeast two hybrid assays were used to validate these RIN3 interacting partners. Live imaging of mCherry- or GFP-tagged constructs was used to examine axonal transport of APP, BACE1, and immunoblotting was used to detect APP processing under the setting of upregulating either RIN3, or BIN1, or CD2AP expression. $Rab5^{S34N}$ (dominant negative variant) was overexpressed to block Rab5 activity. **Results:** RIN3 mRNA level started elevating from embryonic day 18 in primary APP/PS1 BFCNs. Hippocampus RIN3 started to increase from 3-month-old and cortex RIN3 level elevated from 6-month-old. Upregulated RIN3 in primary BFCNs induced early endosome enlargement. BIN1 and CD2AP, another two LOAD risk factors, were identified by ip-MS. They were proved to interact and colocalize with RIN3 via multiple methods. They were recruited to early endosome rather than late endosome or recycle endosome by RIN3 PRD domain. BIN1 neuronal isoform was decreased in 3-month-old APP/PS1 mice brain, while its ubiquitous isoform was increased. CD2AP protein level just mildly increased. Overexpression of RIN3 and CD2AP promoted APP cleavage and produced more APP CTFs in PC12 cells, while upregulating BIN1 neuronal isoform mildly reduced APP-CTFs production. **Conclusion:** We have discovered that the RIN3 AD risk factor was significantly unregulated that correlated with endosomal dysfunction in neurons of APP/PS1 mouse model of AD. We have also identified that RIN3 recruited BIN1 and CD2APto early endosomes. RIN3 and CD2AP impaired axonal transport of APP and BACE1 in axons and dendrites, enhancing beta-cleavage of APP to increase toxic APP CTFs. Our study has thus defined an important role for RIN3 contributing to AD pathogenesis.

P1298/B428

Spatial and Temporal Intracellular Trafficking of Vascular Endothelial Growth Factor (VEGF) Receptors in Microvascular Endothelial Cells.

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Introduction: the vascular endothelial growth factor (VEGF) family is critical for both increased vascular permeability and pathological angiogenesis in a vast number of neovascular diseases like retinopathies. Our group has shown that VEGFRs can shape the neovascular phenotype of vascular endothelial cells when translocated to the nucleus. However, the mechanism of the intracellular trafficking route by which these receptors translocate to and from the nucleus remains unclear. **Aims: to** determine the effect of VEGF on the spatial and temporal translocation of VEGF receptors within microvascular endothelial cells. **Methods:** Primary human retinal microvascular endothelial cells (HREC) were obtained from 2 donors. Near confluent cells were washed 2 times and maintained without growth factors in serum-free medium for 45 minutes. Subsequently, 100ng/ml VEGF165 was added and cells were fixed at 0, 10, 30 and 120 minutes. Cells were immunostained to colocalize VEGFR1 and VEGFR2 with the nucleus, lysosomes, early endosome, trans Golgi, and endoplasmic reticulum (ER). Images were captured by confocal microscopy and the analyses performed on NIS elements and Imaris Software. **Results:** Both VEGFR1 and VEGFR2 were observed at relatively low levels in the nucleus, lysosomes, early endosome, trans Golgi, and ER of control cells not treated with VEGF. Between10 and 30 minutes following VEGF treatment a significant and maximal increase in VEGFR1 and VEGFR2 levels (40 and 60% respectively) were observed in the nucleus when compared to control cells. This increase was associated with a 60% increase in early endosomes and their colocalization with VEGFR2 together with spatial differences in plasma membrane and perinuclear colocalization. Interestingly, the time to reach maximal translocation of receptors to the nucleus was donor dependent. In addition, the increased levels of VEGF receptors in the nucleus were associated with trans Golgi and ER increase (80 and 70% respectively) compared to control. However, this did not change the colocalization coefficients between VEGFR2 and these organelles. By two hours following VEGF treatment, nuclear VEGFR levels had returned to that seen in untreated control cells. At the 2 hour time point a significant, 160% increase in lysosomes was observed in VEGF-treated cells and this increase was associated with increased colocalization with VEGFR2 (Pearson Coefficient >0.9). **Conclusion**: Our data indicates that VEGF stimulation of microvascular endothelial cells activates transcellular transport of VEGFRs to the nucleus via early endosomes and that nuclear VEGFRs are exported and degraded by the lysosomal pathway. The origin of the nuclear VEGFRs appears to be from a combination of plasma membrane receptors and intracellular receptors.

P1299/B429

Creg1 Promotes Endocytic Trafficking and Lysosomal Biogenesis.

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CREG1 promotes endocytic trafficking and lysosomal biogenesis Joshua Chao, Jie Liu, Yanmei Qi, Leonard Y. Lee, Shaohua Li Department of Surgery, Rutgers University-Robert Wood Johnson Medical School, New Brunswick, NJ 08903 CREG1 is a small glycoprotein with a physiological function that remains largely unknown. Contradictory results were reported on its subcellular localization, which is

likely because the antibodies used in those studies were not validated for immunostaining. By using cells overexpressing Myc-tagged CREG1 and tissues of Creg1 knockout mice, we identified two commercially available CREG1 antibodies suitable for immunofluorescence. Using these antibodies, we demonstrate that CREG1 is localized to the early endosome, late endosome, and lysosome in both cultured cells and mouse tissues. Overexpression of CREG1 in hepatocytes enhances macropinocytosis and clathrin-dependent endocytosis as assessed with TRITC-dextran and Alexa 488-transferrin, respectively. This is accompanied by increased expression of lysosomal membrane proteins, proteases, and vacuolar ATPases. As a consequence, amino acid starvation-induced autophagy is significantly augmented. In contrast, deletion of mouse Creg1 in embryonic yolk sac as well as shRNA-mediated knockdown of CREG1 in hepatocytes inhibit endocytosis, reduce lysosomal protein expression, and suppress autophagy. These results demonstrate that CREG1 is an endosomal-lysosomal protein and promotes endocytic trafficking and lysosomal biogenesis.

P1300/B430

Regulation of Osteoclast Function by the Small Gtpase Arl8b.

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Recent studies have established Arl8b-mediated positioning of lysosomes and lysosomes-related organelles as a crucial factor regulating amino acid sensing, cell migration and metastasis, NK cell-mediated cytotoxicity and antigen presentation. Arl8b mediates lysosomal transport to the cell periphery by recruiting its effector, SKIP, which in turn recruits the motor protein kinesin-1 on lysosomes. Arl8b also binds to the Rab7 effector- PLEKHM1, and this interaction repositions Arl8b-positive lysosomes to the perinuclear region of the cell and promotes autophagosomes-lysosome fusion. Interestingly, frameshift mutations in PLEKHM1 result in Osteopetrosis where the bone resorbing functions of osteoclasts is impaired. Osteoclasts resorb bone by secreting their lysosomal contents within the confines of a sealing zone between themselves and the bone surface. Here, we have explored the role of Arl8b in bone remodeling. Arl8b showed a striking localization beneath the actin rings and ruffled borders in osteoclasts, and lysosome secretion was significantly impaired in Arl8b-deficient osteoclasts. Further, unlike control osteoclasts, lysosomes in Arl8b-deficient osetoclasts failed to localize beneath the actin rings or in the ruffled borders. Taken together, our findings establish Arl8b as a crucial component of osteoclast-mediated bone remodeling.

P1301/B431

The Parkinson' Disease Kinase Lrrk2 Promotes Lysosomal Tubulation and Sorting in Ruptured Lysosomes.

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Mutations in LRRK2 are a main cause of familial and sporadic Parkinson' disease (PD). Unfortunately, the role of LRRK2 in the cell remains elusive which heavily limits our therapeutic options. We observed that LRRK2 is recruited to a very specific subset of lysosomes in mouse primary astrocytes. Using an unbiased proteomic screening, we identified JIP4 as a LRRK2 interactor in the lysosomal membrane. Since JIP4 has been linked to stress response and LRRK2-positive lysosomes show variable levels of Cathepsin B (CTSB), we hypothesized that the LRRK2:JIP4 complex responds to lysosomal membrane damage. By treating

astrocytes with LLOME (a reagent that permeabilizes the lysosome), we observed a massive increase in LRRK2 recruitment to the lysosomal membrane in a seemingly lysophagy-independent manner. LRRK2 goes to ruptured lysosomes as observed by the lack of LysoTracker and Magic-red CTSB staining. LRRK2 recruits JIP4 to ruptured lysosomes through its kinase activity, since a blockage of active LRRK2 by pharmacological kinase inhibition or using Lrrk2-KO cells impairs the lysosomal recruitment of JIP4. Additionally, unbiased proteomics reveals the presence of RAB10 and RAB35 (known LRRK2 substrates) in LRRK2-positive lysosomes. LRRK2 recruits and phosphorylates both RAB GTPases in the lysosomal membrane, which leads to the subsequent recruitment of JIP4. Using super-resolution microscopy, we detected JIP4-positive tubules associated with lysosomes which could potentially link LRRK2 to lysosomal sorting. Interestingly, JIP4-positive vesicles seem to associate with other lysosomes, which could imply a form of lysosome-lysosome communication. We thus identified a newly described function of LRRK2 promoting lysosomal tubulation and content release, which can potentially have implications in lysosomal homeostasis and PD pathobiology.

P1302/B432

Putative Phase Separation of the Yeast Adaptor Protein 3 Complex.

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The delivery of proteins to cellular compartments is critical for maintaining organelle identity and cell function. Adaptor protein complexes play a vital role in membrane trafficking by selectively packaging cargo proteins into nascent transport vesicles. In budding yeast, adaptor protein 3 (AP-3) facilitates transport of cargo from the late Golgi to the vacuole. While the basic function of AP-3 has been characterized, less is known about the mechanism of AP-3 recruitment and vesicle formation. For instance, it is unclear if yeast AP-3 associates with coat proteins or how AP-3 might stimulate vesicle budding without a coat. Yeast AP-3 localizes to punctate structures that are adjacent to late Golgi elements. We hypothesize that AP-3 structures are not membrane-bound, and may instead represent liquid-like protein droplets in the cytoplasm. To test this idea, we identified an intrinsically disordered region (IDR) in a subunit of yeast AP-3. This IDR is necessary for normal AP-3 function and localization. When fused to oligomeric proteins in the yeast cytoplasm, the AP-3 IDR is sufficient to drive the formation of foci. These foci exhibit properties characteristic of dynamic liquid droplets: they recover fluorescence rapidly after photobleaching, fuse in live cells, and display temperature sensitivity. Our working hypothesis is that the propensity of the IDR to form liquid droplets drives the phase separation of AP-3. The biological role of this behavior remains to be determined. Recent work has shown that disordered domains of peripheral membrane proteins can sense and stimulate membrane curvature. The necessity of the IDR for AP-3 recruitment and function raises the possibility that the AP-3 IDR may curve membranes into budding vesicles in the absence of coat proteins. Alternatively, the AP-3 IDR may promote clustering in order to sequester excess adaptor or to supply adaptor efficiently to transport machinery. Ongoing experiments will explore these possibilities.

P1303/B433

Synaptic Vesicles Precursors and Lysosomes Use Distinct Machinery for Axonal Transport.

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The ability of lysosomes to move throughout the cytoplasm is critical for various cellular functions such as autophagy, cell migration, and tumor invasion. This ability is particularly crucial in neurons because of their extreme asymmetry and length of axons and dendrites. We recently discovered an eight-subunit complex named BORC that recruits the small GTPase ARL8, the adaptor protein SKIP, and kinesins-1 and -3 to lysosomes, promoting their movement toward the plus-end of microtubules in the periphery of non-polarized cells. Knock-out (KO) of any of the BORC subunits causes clustering of lysosomes in the pericentrosomal area of the cell. Although no human mutations in the BORC subunits have been reported to date, single nucleotide polymorphisms causing altered expression of the diaskedin (BORCS7) subunit of BORC have been associated with schizophrenia, emphasizing the importance of understanding the function of BORC in neurons. In this regard, a study by another group using *C. elegans* showed that mutation of the myrlysin (BORCS5) subunit of BORC impairs axonal transport of synaptic vesicle precursors (SVPs). To analyze the requirement of BORC for transport of lysosomes and SVPs in mammalian neurons, we used the CRISPR/Cas9 system to generate a myrlysin-KO mouse. We found that the resulting KO embryos grew to term, but the pups died immediately after birth. Pathology analyses identified the cause of death as the inability of the pups to breathe, a problem that could be due to defects in the lungs or the nervous system. To address the importance of BORC in neuronal organelle transport, we prepared cultures of hippocampal neurons from control and myrlysin-KO embryos. The neurons were transfected with plasmids encoding lysosomal and synaptic vesicle markers fused to fluorescent proteins. We found that myrlysin-KO prevented transport of lysosomes into the axon but not the dendrites. In contrast, there was no effect on the transport of SVPs into the axon. An alysis of brain tissues, and neuromuscular junctions at the diaphragm, from WT and KO embryos, confirmed that BORC is necessary for lysosomes, but not for SVPs, to enter the axon and to reach synaptic terminals. These findings indicate that in mouse neurons BORC plays a role in transport of lysosomes but not SVPs into the axon. Neuropsychiatric disorders associated with altered BORC expression could thus result from impaired lysosome transport into the axon.

P1304/B434

Lysosomal Functions Play a Critical Role in Restoring Cellular Cholesterol Homeostasis and Quality Control in NPC1-deficient Cells.

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Niemann-Pick type C (NPC) disease is a fatal neurodegenerative disorder caused by mutations in *NPC1* and *NPC2* genes that result in cholesterol accumulation in lysosomes and a deficiency in cellular quality control. Currently, no FDA-approved therapies exist for NPC and the majority of children with NPC die in adolescence. How the NPC1/2 proteins are involved in the trafficking of lysosomal cholesterol and how the defect in lysosomal cholesterol export leads to neurodegeneration remain largely unknown. Using cell biological and biochemical approaches, we attempted to explore the mechanisms of NPC disease. 2-hydroxypropyl-β-cyclodextrin (HPβCD) is known to rescue lysosomal cholesterol accumulation and is undergoing testing in phase 2/3 clinical trials for NPC disease. However, HPβCD's ototoxicity has been a

major concern in moving it forward as a therapeutic agent. Our recent study and the reports from other laboratories showed that 2-hydroxypropyl-γ-cyclodextrin (HPγCD) alleviates cholesterol accumulation in NPC1-deficient cells in spite of its low binding affinity for cholesterol. To understand the mechanisms of NPC disease, we explored the cellular changes that are induced upon HPγCD treatment in NPC1 patient-derived fibroblasts. Here, we show that cell treatment with HPγCD results in the enhancement of lysosomal functions and an improvement in cholesterol homeostasis and cellular quality control. Interestingly, HPγCD administration induced the activation of the transcription factor EB (TFEB), a master regulator of lysosomal biogenesis and autophagy. Furthermore, our study indicates that HPγCD treatment restores the homeostasis of the lysosomes and endoplasmic reticulum and an increase in autophagy in NPC1-deficient cells. In summary, our study suggests that lysosomal functions play a critical role in restoring cellular cholesterol homeostasis and quality control in NPC1-deficient cells Understanding the HPγCD-induced cellular pathways or proteins could assist in developing effective therapies for NPC disease.

P1305/B435

The Gator Complex Regulates the Lysosomal Dynamics of the Torc1 Inhibitor Tsc.

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Tuberous sclerosis is a rare multiorgan genetic disorder affecting 1 in 6000 newborns per year. It is caused by mutations in the genes of the subunits of the Tuberous sclerosis complex (TSC), one of the inhibitors of the Target of Rapamycin Complex I (TORC1). Mutations in TSC components result in the hyperactivation of TORC1, which causes the growth of benign tumors in many parts of the body. Although the importance of the TSC in cell metabolism has been well established, how it is regulated remains poorly understood. In this study, we use the experimental of *Drosophila melanogaster* and tissue culture cells, to show that the Gap Activity Towards Rags (GATOR) complex is a novel regulator of TSC. The GATOR complex acts as an upstream regulator of TORC1. It contains two subcomplexes. The GATOR1 complex inhibits TORC1 in response to amino acid starvation. The GATOR2 complex promotes TORC1 activity by inhibiting the function of GATOR1. GATOR1 inhibits the activity of TORC1 by preventing the recruitment of the complex to lysosomes where it encounters its activator Rheb. Specifically, GATOR1 regulates TORC1 localization by serving as a GTPase activating protein for RagA/B, which in their GTP bound status, recruit TORC1 to lysosomes. Using Fluorescence Recovery After Photobleaching (FRAP) we determined that knocking out WDR24, one of the subunits of the GATOR2 complex, resulted in the increased recruitment of the TSC subunits TSC2 and TSC1 to lysosomes, both in Hela cells and in *vivo* in the *Drosophila* ovary. Furthermore, we demonstrated that the GATOR2 complex regulates TSC2 dynamics through the regulation of the guanine nucleotide binding status of the RagA or RagC small GTPases. Specifically, the GDP bound RagA and the GTP bound RagC promotes the recruitment of TSC2 to lysosome. Moreover, by using a photoconvertible protein tagged TSC2, we determined the fast binding of TSC2 to lysosome in *wdr24*$^{-/-}$ cell is accompanied by its rapid dissociation from lysosome. Taking together, we provide both in *vitro* and in *vivo* evidence to support that the GATOR complex regulates TSC's dynamic cycling between lysosome and cytoplasm and its ability to inhibit TORC1 activity.

P1306/B436

Fast Targeting of Antigen and Surface-derived MHCII into Degradative Compartments Implies Endosomal Prewiring for Antigen Presentation in B Cells.

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In order to mount high-affinity antibody responses, B cells internalise specific antigens and process them into peptides loaded onto MHCII for presentation to T_H cells. While the biochemical principles of antigen processing and MHCII loading have been well dissected, how the endosomal vesicle system is wired to enable these specific functions remains much less studied. Here, we performed a systematic microscopy-based analysis of antigen trafficking in B cells to reveal its route to the MHCII peptide-loading compartment (MIIC). Surprisingly, we detected fast targeting of internalised antigen into peripheral acidic compartments that possessed the hallmarks of MIIC and also showed degradative capacity. In these vesicles, internalised antigen converged rapidly with membrane-derived MHCII and partially overlapped with Cathepsin-S and H2-M, both required for peptide loading. These early compartments appeared heterogenous and atypical as they contained a mixture of both early and late markers, indicating specialized endosomal route. Together, our data suggests that, in addition to previously-reported perinuclear late endosomal MIICs, antigen processing and peptide loading could start already in these specialized early peripheral acidic vesicles to support fast peptide-MHCII presentation.

P1307/B437

USP19 Cooperates with DNAJC5 to Promote Unconventional Secretion of Misfolded Cytosolic Proteins through Non-Degradative Endolysosomes.

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Elimination of misfolded proteins is essential for the integrity of the protein homeostasis network and for cell viability in eukaryotic cells. Misfolding-associated protein secretion (MAPS) is an unconventional protein-disposing mechanism that specifically exports misfolded cytosolic proteins including some neurodegenerative disease-causing proteins such as alpha-Synuclein and TAU. However, how MAPS cargos are selected for release into the cell exterior is unclear. Here we establish a deubiquitinase (USP19)-chaperone (DNAJC5) axis as a 'triaging hub' for MAPS cargos. USP19, a previously established MAPS regulator binds HSC70 at the surface of the endoplasmic reticulum (ER), which collectively recruits misfolded proteins to the ER surface. This event occurs upstream of DNAJC5, a HSC70 co-chaperone preferentially localized to the surface of late endosomes/lysosomes. Following substrate recruitment to the ER membranes, DNAJC5 can chaperone MAPS client proteins into the lumen of endolysosomes, which are often in close contact the ER network. Misfolded proteins are then secreted from late endosomes presumably because vesicles derived from late endosomes can fuse with the plasma membrane. We further determined the mechanism by which DNAJC5 translocates MAPS cargos into the endolysosomal lumen, which is distinct from the previously established chaperone-mediated autophagy (CMA) pathway because unlike the CMA pathway, the secretion of misfolded proteins in MAPS does not require cargo unfolding, is inhibited by serum starvation, and is not dependent on the CMA motif in cargos. Finally, upon secretion, misfolded proteins can be taken up by recipient cells through endocytosis and eventually degraded in the lysosome. We conclude that MAPS is a novel protein quality control mechanism that promotes protein homeostasis by exporting misfolded proteins to cell exterior

when excessive proteotoxic stress overwhelms the protein homeostasis network. However, when mis-regulated, this process may contribute to the intercellular transmission of misfolded proteins that are often associated with the progression of many neurodegenerative diseases.

P1308/B438

Regulation of Vacuolar Membrane Integrity by a Protein Trafficking Complex in Yeast Cell Death.

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Fungal pathogens are a growing concern with an estimated 1 billion infections and 1.5 million deaths annually. In addition, plant fungal pathogens increasingly threaten global food sources. Limited treatment options and rising resistance to available drugs underscore the need for development of new approaches to combat this growing public health threat. The topic of microbial cell death has been controversial, but growing evidence on several fronts has demonstrated, at least for some fungi, cell death is required (e.g. *Magnaporthe* and *Podospora*). Elucidation of previously unrecognized cell death mechanisms using *Saccharomyces cerevisiae* as a tool could provide a new platform for the development of novel therapeutics. Using a genome-wide screen, we have identified a clathrin adaptor protein trafficking complex (AP-3) as a critical factor in limiting yeast survival after thermal stress; deletion of any AP-3 subunit results in profound increased survival following some but not all types of stress. The complex has a conserved established function in sorting protein cargo for vesicular trafficking from the Golgi apparatus to the yeast vacuole/lysosome and its role in regulating cell death in yeast after stress appears to require this trafficking function. We show that heat induced death is preceded by vacuolar permeabilization and that loss of AP-3 function protects cells. These results are consistent with a hypothesis that AP-3 sorts “killer” proteins that control stress-induced vacuolar lysis.

P1309/B439

Single Cell an alysis of Vacuolar Ph Using Confocal Microscopy.

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In order for ion storage, protein degradation and protein folding to occur properly, vacuolar pH must lie within a specific range. Due to the breadth of the vacuoles function, pH regulation has direct implications in cellular age. This impact could provide insight into human diseases such as lysosomal storage diseases and Alzheimer’s disease. Despite this, there have been few studies to directly observe to what extent vacuolar pH impacts other vacuolar phenotypes. In our previous work, we have observed vacuolar pH via confocal microscopy to collect single-cell data using the pH-sensitive vacuolar dye, BCECF-AM. However, the use of this dye resulted in data with high levels of variance, which has led to inconclusive results. To rectify this, we have developed a fluorescent protein tag that utilizes an eGFP-mCherry double tag, which allows us to measure vacuolar pH without the use of dyes. This tag works similar to other pH-sensitive dyes, leading us to hypothesize that our protein tag will yield similar results to our previous findings. In this work, we compare the effectiveness of this protein tag to our previous findings with BCECF-AM. Our preliminary results show that this protein tag has a similar pH indicator range to other pH probes, and is more stable than other probes, highlighting its advantages as a pH indicator. Taken together, our findings show that our double-tag indicator has the potential to provide

better quality data, which will allow us to further understand the impact of vacuolar pH on other cellular functions.

ER and Golgi Transport

P1310/B440

Mutations in the core-TRAPP subunit *TRAPPC4* are associated with severe syndromic intellectual disability and affect both membrane traffic and autophagy.

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The transport protein particle (TRAPP) complexes II and III regulate key trafficking events and TRAPP III is also required for autophagy. TRAPPC4 is a core component of the TRAPP complexes and one of the essential subunits for guanine nucleotide exchange factor activity. Pathogenic variants in specific TRAPP subunits are associated with neurological disorders. We undertook whole exome sequencing in three unrelated families of Caucasian, Turkish and French-Canadian ethnicities with seven affected children with features of early onset seizures, developmental delay, microcephaly, sensorineural deafness, spastic quadriparesis and progressive cortical and cerebellar atrophy in an effort to determine the genetic etiology. In all seven affected individuals we identified a rare homozygous variant in a non-canonical well-conserved splice site within TRAPPC4 (hg19:chr11:g.118890966A>G; TRAPPC4: NM_016146; c.454+3A>G). In silico analysis predicted the variant to cause aberrant splicing. Consistent with this, we showed both a reduction in full-length transcript and a shorter transcript missing exon 3, suggestive of an incompletely penetrant splicing defect. TRAPPC4 protein levels were significantly reduced and biochemical analysis demonstrated a defect in TRAPP complex assembly and/or stability. Intracellular trafficking demonstrated significantly delayed entry into and exit from the Golgi in fibroblasts derived from one of the affected individuals that was rescued upon lentiviral expression of wildtype TRAPPC4. Consistent with the recent association of the TRAPP III complex in autophagy, we found that the fibroblasts had a basal autophagy defect and a delay in autophagic flux, likely due to unsealed autophagosomes. These results were validated using a yeast *trs23* temperature sensitive mutation that exhibits constitutive and stress-induced autophagic defects at permissive temperature and a secretory defect at restrictive temperature. Our study suggests that reduced levels of TRAPPC4 affect both autophagy and membrane trafficking. This is the first report of a TRAPPC4 disease-associated variant and our findings add to the growing number of TRAPP-associated neurological disorders.

P1311/B441

Efficient Golgi Forward Trafficking Requires GOLPH3-Driven, PI4P-Dependent Membrane Curvature.

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Vesicle budding for Golgi-to-plasma membrane trafficking is a key step in secretion. Proteins that induce curvature of the Golgi membrane are predicted to be required, by analogy to vesicle budding from other membranes. Here, we demonstrate that GOLPH3, upon binding to the phosphoinositide PI4P, induces

curvature of synthetic membranes in vitro and the Golgi in cells. Moreover, efficient Golgi-to-plasma membrane trafficking critically depends on the ability of GOLPH3 to curve the Golgi membrane. Interestingly, uncoupling of GOLPH3 from its binding partner MYO18A results in extensive curvature of Golgi membranes, producing dramatic tubulation of the Golgi, but does not support forward trafficking. Thus, forward trafficking from the Golgi to the plasma membrane requires the ability of GOLPH3 both to induce Golgi membrane curvature and to recruit MYO18A. These data provide fundamental insight into the mechanism of Golgi trafficking and into the function of the unique Golgi secretory oncoproteins GOLPH3 and MYO18A.

P1312/B442

Utilizing Crispr/Cas Editing to Tag Endogenous COL1A2 in Order to Visualize Intracellular Trafficking and Quality Control of Procollagen in Osteoblasts.

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The intracellular trafficking of type 1 collagen, despite being the most abundant protein in the human body, is still poorly understood. Procollagen has been shown to accumulate in the ER and disruptions in procollagen secretion are implicated in several connective tissue disorders. The mechanisms underlying these defects and the resulting cell stress remain unclear. The mechanisms of procollagen secretion and quality control have been probed using transient overexpression of fluorescently- tagged procollagen chains. Live cell imaging revealed many unknown features of procollagen trafficking including an unexpected composition of vesicles trafficking procollagen from ER to Golgi as well as a non-canonical autophagic pathway that redirects misfolded procollagen from ER exit sites to lysosomal degradation. Transient transfections are limited in temporal resolution as well as have varying levels of expression per cell. We are limited in the questions that can be answered using this tool. Here, we used CRISPR/Cas to knock in GFP cDNA into the endogenous *Col1a2* gene in an MC3T3 mouse osteoblast cell line, replacing a stretch of gDNA from the middle of exon 2 to the middle of exon 6 to retain endogenous splice sites. In the resulting proα2(I) chain of type I procollagen, GFP replaces most of the N-terminal propeptide. Unique FRT sites were placed in introns 1 and 6 of *Col1a2* to enable *Flp*-recombinase mediated cassette exchange (*Flp*-RMCE) of the GFP cDNA with other tags. Several heterozygous clones were obtained. We identified the clones that produced normal heterotrimers of type I procollagen and formed a bone-like mineralized matrix for use in future studies. We are currently using a similar technique to introduce common pathogenic mutations in *Col1a2* to create cell lines that could be used for systematic studies of the pathophysiology of osteogenesis imperfecta mutations. We are also using the cell line to investigate the role of various genes implicated in new cases of skeletal dysplasia like *Creb3L1*.

P1313/B443

Uncoating of CopII from Endoplasmic Reticulum Exit Site Membranes Precedes Cargo Accumulation and Membrane Fission.

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COPII and COPI are considered to be analogous sets of vesicle coat protein heterocomplexes. Coupled to cargo selection, they mediate the formation of membrane vesicles translocating in opposite directions

to different destinations within the secretory pathway. Here, live cell imaging and electron microscopy provided direct evidence for a different localization and mode of function of the COPII coat during protein export from the endoplasmic reticulum (ER). We have studied the ER export of fluorescently tagged Vesicular Stomatitis Virus G as well as CFTR and procollagen I. Pharmaceutical and genetic perturbations of ER-Golgi transport were used to demonstrate that COPII is recruited to membranes defining the boundary of ER-ER Exit Sites (ERES). Within this ER-ER Exit Sites boundary the COPII heterocomplex facilitates selective cargo concentration in ERESs membranes. Thus, the uncoating of COPII from ERESs membranes precedes cargo accumulation and the budding of Golgi-bound carriers. Moreover, we report what may be direct transfer of cargo to the Golgi apparatus from Golgi-associated BFA-sensitive subpopulation of ERESs. Finally, in *ldlF* cells the stably expressed functional epsilon-COPI-EYFP labeled both ERESs and anterograde carriers. Thus, we demonstrate an alternative model of how the COPII complex orchestrates the functions of the ERES domain. We propose two distinct mechanisms that together facilitate the selective cargo entry into ERESs. The first is the known cytosolic cargo export motif interaction with the COPII Sec24subunit. The second is based on alleviation of mismatching interactions of cargo protein TMD with its surrounding membrane. These findings change our understanding of the role of coat proteins in ER to Golgi transport.

P1314/B444

ALS/FTD-linked UBQLN2 Mutations Result in Aberrant Serpin Accumulation.

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Missense mutations in *UBQLN2*, encoding a protein that functions in protein quality control, cause X-linked dominant amyotrophic lateral sclerosis (ALS) with frontal temporal dementia (FTD). However, the mechanism(s) by which the mutations cause disease remains unclear. To gain insight into the pathophysiologic mechanisms driving disease, we generated lines of transgenic (Tg) mice expressing either wild-type human UBQLN2 (WT356 line) or carrying the P497S ALS/FTD mutation (P497S line). The P497S line recapitulated key features of the human disease, including development of age-dependent motor neuron disease, cognitive impairments, and pathologic accumulation of UBQLN2 inclusions in the spinal cord and brain. These symptoms and pathology were largely absent in the WT356 line, despite equivalent transgene expression. A comparison of the proteomic changes in the hippocampus and spinal cord of the animals revealed several members of the serpin protein family as being more highly expressed in P497S animals compared to either WT356 or Non-Tg animals. Serpins are a diverse group of intracellular and secreted proteins that function primarily in serine protease inhibition. They entrap and destroy proteases through a conformational switch of the protein. However, this metastability makes them highly prone to misfolding and polymerization, causing diseases termed serpinopathies. We examined the possibility that ALS/FTD mutations in UBQLN2 perturb proteostasis causing serpins to misfold. Indeed, double immunofluorescent confocal microscopy revealed colocalization of Serpin A1 and C1 with UBQLN2 inclusions that form in the hippocampus and spinal cord of P497S mutant mice, while Serpin I1 formed inclusions independent of UBQLN2 colocalization. Additionally, serpins A1, B1, C1 and I1 all showed an age dependent accumulation. To investigate the aberrant aggregation of serpins we conducted GST-pulldown assays examining whether UBQLN2 binds serpins. We also studied the effects of CRISPR/Cas9-targeted inactivation of UBQLN2 on serpin aggregation. Our findings indicate UBQLN2 binds directly with serpin proteins, and that ALS/FTD mutations in UBQLN2 enhance binding with the proteins. Furthermore, knockout of UBQLN2 expression in HeLa and NSC motor neuron cells

resulted in increased aggregation of serpins. These studies are consistent with ALS/FTD mutations in UBQLN2 causing aberrant serpin accumulation through both a gain- and loss-of-function mechanism. Taken together, our studies have identified a novel function of UBQLN2 in proper regulation of serpin expression and show that misaggregation of serpin proteins is a pathologic consequence of UBQLN2 mutations.

P1315/B445

Bad-lamp Controls Human Tlr9 Access To, and Generation Of, Signaling Endosomes in Plasmacytoid Dendritic Cells in Response to Innate Sensing.

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Toll-like receptors (TLR) are essential components of the innate immune system. Several accessory proteins, such as UNC93B1, are required for transport and activation of nucleic acid sensing Toll-like receptors in endosomes We have demonstrated[1] that, upon microbial activation of human plasmacytoid dendritic cells (pDC) BAD-LAMP (LAMP5) controls traffic and activatory processing of TLR9 via an unconventional secretion route, from ERGIC to LAMP1+ late endosomes. As a result, a fine-tuned bimodal signaling leads first to IRF-driven Interferon type I production, rapidly replaced by NF-κB -driven pro-inflammatory response. The specific and sequential recruitment of signaling complex components occurs thanks to the appearance of a VAMP3+/LAMP2+/LAMP1− endolysosome compartment. BAD-LAMP-silencing enhances TLR9 retention in this post-ERGIC compartment and consequent downstream signaling events. Conversely, sustained BAD-LAMP expression in pDCs contributes to their lack of type I IFN production after exposure to a TGF-β-positive microenvironment or isolation from human breast tumours. Hence, BAD-LAMP limits interferon expression in pDCs indirectly, by promoting TLR9 sorting to late endosome compartments at steady state and in response to immunomodulatory cues. 1.Combes et al.(2017) *Nature Communications*, *8*(1), 913. http://doi.org/10.1038/s41467-017-00695-1

P1316/B446

Regulation of Protein Transport into the E.R. By Phosphorylation of the β-subunit of the Sec61 Channel.

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The beta subunit of the Sec61 channel is phosphorylated in both yeast and mammals. We asked whether phosphorylation of yeast Sbh1p is used to modulate channel activity as a fast response to acute needs. In the cytosolic domain of yeast Sbh1p, 7 sites have been found phosphorylated in different combinations in phosphoproteome screens. At the Sbh1p N-terminus there are 2 proline flanked phosphorylation sites -S3 and T5-, of which T5 is conserved in mammalian Sec61β. Neither is present in yeast Sbh2p, which is the β subunit of the Sec61 homologous Ssh1 channel. The aims of my project are to identify the effects of Sbh1p phosphorylation on Sec61 channel functions and the kinases responsible for this modification. In addition, I have been identifying the proteins whose ER import is affected by Sbh1p phosphorylation, to characterize their ER targeting sequences. Sbh1p is the only non-essential subunit of the Sec61 channel. To identify ER translocation substrates dependent on the presence of Sbh1p and the substrates whose ER import depends on Sbh1 phosphorylation, I performed an automated microscopic screen in yeast on ER import or membrane insertion of 400 secretory and transmembrane proteins tagged with GFP. At the same time, for the identification of the kinase and

phosphatase responsible for phosphorylation and dephosphorylation of Sbh1p we performed a microscopic screen on all yeast kinase and phosphatase knockouts and overexpression mutants expressing a GFP-tagged, Sbh1p phosphorylation-dependent substrate. We were able to identify 45 Sbh1p-dependent ER translocation substrate, as well as 7 Sbh1p phosphorylation-dependent ER translocation substrates, which were also Sbh1p-dependent. Sbh1-dependence was confirmed by Western Blotting for cytosolic precursors in sbh1 mutant strains. We have found that signal peptides dependent on Sbh1p for ER import had less polar C-regions than generic signal peptides. Transmembrane domains (TMD) dependent on Sbh1p had strong polybasic patches to one side of the TMD. Sbh1p-phosphorylation-dependent targeting sequences were subsets of these, whose features we are currently characterizing both by bioinformatics and experimentally. We were also able to identify 13 potential kinases responsable for the phosphorylation of Sbh1p, and we are optimizing a method, using Phos-Tag gels, to enable us to identify by differential gel migration the kinase and phosphatase responsable for modifying Sbh1p. We also raised an antibody against the S3-phosphorylated N-terminus of Sbh1p which allows us to analyze the conditions leading to this modification. This work will allow us to understand the feedback loop between biosynthetic protein import into the ER, the UPR, and kinase signaling.

P1317/B447

An Optical Assay to Study Proteotoxic-induced Chromosome Duplication.

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The Hampton lab has recently shown that the ER-resident rhomboid derlin Dfm1 is required for removal of misfolded proteins from the ER membrane. In cells lacking Dfm1, misfolded proteins accumulate and cause proteotoxic stress. In response, cells undergo an alternative clearance mechanism for misfolded proteins through aneuploidy-induced duplication of chromosome XV. To establish a readout of chromosome XV duplication, we have integrated green fluorescent protein (GFP) optical reporter onto chromosome XV. Cells with an extra copy of chromosome XV exhibit double the fluorescence of those with a single copy. Accordingly, we are able to use this as a tool to study how yeast use chromosome duplication, as indicated by colony fluorescence, to overcome cellular stress caused by misfolded proteins. This strain allows for a straightforward optical assay to genetically define components of the stress response in Dfm1-deficient cells. In this way, we aim to characterize mechanisms responsible for this novel form of protein quality control in the ER membrane.

P1318/B448

Investigating the Trafficking of Glycosylation Enzymes within the Golgi Apparatus.

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The Golgi apparatus is a major site of glycan processing in the cell which is carried out by a large family of Golgi-resident glycosylation enzymes. These enzymes are generally type II transmembrane proteins with short cytosolic tails at their N-terminus, relatively short transmembrane domains (TMD) and a disordered lumenal ‘stem’ region followed by a catalytic domain. Recent work has suggested the tail and TMD operate via distinct mechanisms to promote the packaging of enzymes into retrograde COPI vesicles to facilitate Golgi retention. It is proposed that it is energetically favourable for the short TMDs of enzymes to segregate into the compatibly thin membranes of budding COPI vesicles. Meanwhile, the

tails of only a handful of enzymes have been shown to interact with COPI coats directly or indirectly via the putative COPI adaptor GOLPH3. What is not clear is the extent and relative contribution of each proposed mechanism to Golgi retention. Using affinity chromatography and mass spectrometry, we confirm GOLPH3 and close relative GOLPH3L can interact with COPI and we also identify a plethora of novel potential GOLPH3+3L clients. Using LOPIT-DC spatial proteomics, we demonstrate that in GOLPH3+3L double knockout U2OS cells a number of these clients are mislocalised from the Golgi to the lysosome for degradation. Cell surface lectin stains show that knockout cells exhibit global defects in glycosylation. We use a flow cytometry-based in *vivo* Golgi retention assay to demonstrate that the tail of a novel client is sufficient for the Golgi retention of a plasma membrane reporter in a GOLPH3-dependent manner. Furthermore, using the same assay we show that the short TMD of the same enzyme is also sufficient for Golgi retention in a more robust, GOLPH3-independent manner. In conclusion, we show that the tail and TMD of Golgi enzymes promote Golgi retention via distinct, complementary and additive mechanisms. We also identify and validate a vast array of novel GOLPH3+3L clients and in doing so, demonstrate that GOLPH3+3L are important for the trafficking of a large proportion of Golgi-resident transmembrane proteins including Golgi enzymes.

P1319/B449

Conditional Genetic Inactivation of Nonmuscle Myosin II in Mouse Renal Epithelium Results in Aberrant Er Processing of Gpi-anchored Protein Uromodulin.

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Conditional genetic inactivation of Nonmuscle Myosin II in adult mouse renal epithelium results in aberrant ER processing of GPI-anchored protein uromodulin. Karla L. Otterpohl, Ishara Ratnayake, Ryan G. Hart, Brook W. Busselman, Phil Ahrenkiel, Kameswaran Surendran and Indra Chandrasekar Nonmuscle myosin II isoforms are actin-associated molecular motors that play key roles in multiple biological processes. We previously demonstrated critical roles for NMII isoforms encoded by the *Myh9* and *Myh10* genes in membrane remodeling during mammalian endocytosis. To identify the roles of NMII proteins in specialized cellular transport pathways, we used mouse kidney as the model system. We generated doxycycline inducible, conditional knockout (cKO) mice in which the *Myh9* (NMIIA) *and Myh10* (NMIIB) genes are simultaneously inactivated in the renal epithelium of adult mice. The Myh9&10 cKO mice show progressive renal tubular dilatation, interstitial hypercellularity and mild fibrosis leading to chronic kidney disease. The *Myh9&10* cKO mouse kidneys show progressive mislocalization and eventual loss of thick ascending limb (TAL) specific, GPI-anchored protein uromodulin and reduced expression of $Na^+K^+2Cl^-$ co-transporter (NKCC2). Histopathology in the cKO animals is similar to that of uromodulin associated autosomal dominant tubulointerstitial kidney disease. In transgenic mouse models, expressing mutant uromodulin, disease progression is driven by increased endoplasmic reticulum (ER) stress caused by retention of the mutant protein in the ER, eliciting an unfolded protein response (Bernascone et.al, 2010). Immunoblot analysis using whole kidney lysates in the Myh9&10 cKO animals showed increased levels of the non-glycosylated, immature form of uromodulin. We hypothesized that loss of Myh9 and Myh10 results in aberrant anterograde trafficking of uromodulin through the ER, resulting in accumulation of uromodulin. Transmission electron microscopy analysis of cKO kidney tissues showed abnormal ER expansion, increased vacuoles and intracellular vesicles. Immunostaining for the ER marker Concanavalin a showed abnormalities in ER

structure in both the proximal and TAL renal epithelial cells in the kidney. Interestingly, immunostaining results showed mislocalization of the N-glycosylated protein chaperone calnexin, only in uromodulin expressing epithelial cells. The unfolded protein response pathway is being evaluated in the cKO mouse model. We conclude that Myh9 and Myh10 proteins play key roles in regulating transport of the GPI-anchored protein uromodulin. Ongoing studies involve evaluating the role of NMII proteins in ER mediated anterograde transport pathways using the cKO mouse models and a novel TAL epithelial cell culture system developed in the lab.

P1320/B450

BioID-based Screening of Biotinylation Sites Using Tamavidin 2-REV Globally Identifies Interactors of Stimulator of Interferon Genes (STING).

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Stimulator of interferon genes (STING), an endoplasmic reticulum (ER)-resident membrane protein, mediates cytosolic DNA-induced innate immune signaling via vesicle-mediated trafficking. After activation by ligand binding, STING moves from the ER through the Golgi apparatus to endosomes and lysosomes. Global identification of proteins that spatiotemporally interact with STING will provide a better understanding of the molecular basis of this organelle translocation and whole signaling networks of STING. The proximity-dependent biotin identification (BioID) method is a powerful technology to identify physiologically relevant protein-protein interactions as well as proximate proteins in living cells. However, biotinylated peptides are rarely detected in the conventional BioID method using streptavidin beads that pull-down biotinylated proteins, because biotin-streptavidin interaction is too strong to elute biotinylated peptides. As a result, only non-biotinylated peptides are identified, which cannot be distinguished from peptides of non-specifically pull-downed false positive proteins. Here we developed a simple method to efficiently and specifically enrich biotinylated peptides using Tamavidin 2-REV, an engineered avidin-like protein with reversible biotin-binding capacity. From RAW264.7 macrophages stably expressing TurboID-fused STING, we successfully identified over 2,500 biotinylation sites on proteins that are proximal to and/or interact with STING. While various ER proteins were biotinylated in unstimulated cells, STING activation caused biotinylation of many proteins localized in the Golgi, endosomes, and lysosomes. These proteins included previously known interactors of activated STING, such as TANK-binding kinase 1 (TBK1) and p62/SQSTM1. Furthermore, interferon-induced transmembrane protein 3 (IFITM3), an endolysosome-localized antiviral protein, bound to STING at the late activation stage. These dynamic interaction profiles may provide a new insight into the STING signaling, and our approach using Tamavidin 2-REV would enable researchers to utilize BioID and other identification methods based on protein biotinylation more effectively.

P1321/B451

Analysis of the Molecular Mechanisms That Regulate Export of a Planar Cell Polarity Protein, Frizzled6, Out of the Endoplasmic Reticulum.

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Planar cell polarity (PCP) is defined as a polarization process in which cells are polarized along the plane of the tissue. PCP plays important roles in many developmental processes and defects in PCP cause various diseases including cancer metastasis. PCP is regulated by signaling receptors which are

asymmetrically localized in cell boundaries. Newly synthesized PCP proteins are delivered along the secretory transport pathway from the endoplasmic reticulum (ER) to the plasma membrane. However, the molecular mechanisms that regulate this transport process remain largely unclear. Here, we analyzed the export of a PCP protein, Frizzzled6, out of the ER. We found that Frizzled6 contains two conserved polybasic motifs: one located on its first intracellular loop and the other located on its C-terminal cytosolic domain. These motifs are important for the interaction between Frizzled6 and the Coat protein complex II (COPII) coat, an important protein complex that regulates packaging cargo proteins at the ER. We found that the Sar1A subunit of the COPII coat directly interacts with the polybasic motif on the first intracellular loop of Frizzled6. A synthetic peptide that corresponded to the first intracellular loop of Frizzled6 blocked vesicular release of Frizzled6. These results demonstrate the importance of the polybasic motif for ER export of Frizzled6. We also identified two N-glycosylation sites on Frizzled6 luminal domain, which are important for ER export of Frizzled6. Moreover, our immunofluorescence data and vesicle immuno- isolation data indicate that another PCP receptor, Celsr1 is in the same vesicle with Frizzled6, and promote surface delivery of Frizzled6, suggesting that these two PCP proteins are associated with each other in the early secretory transport pathway en route to their final destinations. Our results reveal an insight into the molecular machinery that regulates ER export of Frizzled6.

P1322/B452

Characterizing the Role of Jagunal Homolog 1 Protein in Vertebrate Physiology.

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The highly conserved endoplasmic reticulum protein Jagunal (JAGN1) was first identified as a requirement for Drosophila melanogaster oocyte growth. Subsequently, mutations in JAGN1 in human patients with Severe Congenital Neutropenia (SCN) were correlated with defects in neutrophils, including altered granules, diminished fungal-killing capacity, and aberrant glycosylation of proteins. SCN patients also exhibit aberrant bone development and altered neurological and pancreatic functions. Yet, despite its physiological importance, the cellular role of JAGN1 is not known. We chose the zebrafish (*Danio rerio*), and mammalian cells in culture as models to address the general and tissue-specific molecular functions of JAGN1. The zebrafish is an optimal model to study developmental biology of neutrophils and other tissues, as zebrafish larvae are transparent and can be used to visualize transgenically fluorescent tissue development. Zebrafish have two homologs of human JAGN1: JAGN1a and JAGN1b. This provides an additional advantage since each may have a distinct function and define distinct molecular mechanisms of JAGN1 action. We have used CRISPR-Cas9 technology to generate homozygous JAGN1a-/- and JAGN1b-/- knockout fish, and are in the process of elucidating the role of these proteins by monitoring the development of tissues that are affected in patients with JAGN1 mutations: neutrophils, bone, and pancreas. In addition, we are generating JAGN1a/JAGN1b-/- double knockout fish to determine the consequences of eliminating both JAGN1 homologs on early development. We aim to identify the interactors of JAGN1 in mammalian cells using an affinity approach, and have generated the N-terminal and C-terminal regions of JAGN1 as a recombinant protein matrix for pull-downs from mammalian cells. Identifying JAGN1 interactors is necessary to place it within specific pathways and to propose possible functions. Our studies aim to elucidate a specific role for JAGN1 function in vertebrate physiology.

P1323/B453

Sec24 Isoform Specificity Regulates the Assembly of Gamma-secretase from Dimeric Subcomplexes.

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γ-Secretase is a diaspartyl protease with presenilin (PSEN) as the catalytic subunit. It targets a plethora of type I transmembrane proteins and, as such, affects a broad range of physiological processes. Because of this, aberrant functioning of γ-secretase is linked to several diseases including cancer and Alzheimer's disease (AD). γ-Secretase consists, besides PSEN, of nicastrin, APH1 and PEN-2 and this tetrameric nature necessitates a stoichiometric assembly process of which the spatial and temporal regulation remains poorly understood. Cell fractionation combined with blue native electrophoresis shows that subcomplexes are mainly present in the endoplasmic reticulum (ER)-enriched fractions while full complexes appear in the ER-Golgi intermediate compartment, indicating that final assembly occurs beyond the ER. In *vitro* reconstitution of ER export reveals that none of the γ-secretase subunits is required for ER-exit of the others. However, knock-out of any subunit induces the accumulation of preceding subcomplexes in COPII vesicles. Mutating a DPE motif in the cytosolic loop domain of PSEN1, which is lost in the familial AD-associated PSEN1ΔE9 mutant, abrogates ER-exit of PSEN1 as well as PEN-2, but not of nicastrin. This is explained molecularly by the selectivity of Sec24A/B and Sec24C/D in packaging PSEN1 and nicastrin, respectively, and argues against full complex assembly prior to ER-exit. In conclusion, and for the first time, our data support a model wherein the prior formation of dimeric subcomplexes in distinct COPII vesicles combined with active Golgi-to-ER recycling are key events in the assembly of γ-secretase.

P1324/B454

Cryo-Electron Microscopy Characterization of Purified Vap-A Engaged in in *Vitro* Membrane Contact Sites.

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Normal 0 false false false EN-US X-NONE X-NONE /* Style Definitions */ table.MsoNormalTable {mso-style-name:"Table Normal"; mso-tstyle-rowband-size:0; mso-tstyle-colband-size:0; mso-style-noshow:yes; mso-style-priority:99; mso-style-parent:""; mso-padding-alt:0cm 5.4pt 0cm 5.4pt; mso-para-margin-top:0cm; mso-para-margin-right:0cm; mso-para-margin-bottom:8.0pt; mso-para-margin-left:0cm; line-height:107%; mso-pagination:widow-orphan; font-size:11.0pt; font-family:"Calibri",sans-serif; mso-ascii-font-family:Calibri; mso-ascii-theme-font:minor-latin; mso-hansi-font-family:Calibri; mso-hansi-theme-font:minor-latin; mso-bidi-font-family:"Times New Roman"; mso-bidi-theme-font:minor-bidi;} VAMP-associated protein (VAP) is a small, conserved transmembrane protein present in the ER. VAP-A and VAP-B, two isoforms, interact through a FFAT motif with more than 100 cytoplasmic proteins. Many of them, when complexed with VAP-A or VAP-B, form membrane contact sites (MCS) that connect ER to various organelles [1,2]. These narrow cytosolic gaps (<30 nm) are of key importance for intracellular

lipid transport, intracellular signaling, organelle inheritance and lipid metabolism. They have received considerable attention in recent years due to their involvement in certain metabolic diseases. We reconstructed the first functional MCS between ER-like proteoliposomes containing a cytosolic domain of VAP-A and PI4P containing Golgi-like liposomes in presence of the oxysterol binding protein (OSBP). We have shown that in this MCS OSBP is able to transfers cholesterol from ER using the energy associated with counter-transport of PI4P from trans-Golgi apparatus [3,4]. Here, we expressed, purified and reconstituted full length VAP-A in vesicles with variable protein density. Then, we designed an in *vitro* MCS model system to control the formation of contact sites. We combined Cryo-Electron Microscopy and Tomography to describe the architecture of the VAP-A alone or engaged in MCS. Data analysis provided evidence of unexpected features of OSBP and VapA playing an important role in contact formation. First, we shown that the OSBP N-terminus regions act as an entropic barrier to control protein orientation and increase protein dynamic at membrane contact sites [5]. Secondly, Cryo-EM results show a strong flexibility of Vap-A that can span from 8 towards 17 nm depending on local protein density. As a consequence, this behavior enables Vap-A structural adaptation, necessary to form MCS of varying lengths. Moreover, we were able to show that the presence of ORD domain increase disorder distribution of tethers in the MCS regions that might be necessary to allow ORD movements between two facing membranes.

P1325/B455

COPI and COPII Cooperate at ER Exit Sites to Support ER-to-Golgi Protein Trafficking Revealed by 3D Ultrastructure an alyses and Live-Cell Imaging.

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The endoplasmic reticulum (ER) is the site of many important cellular processes, among them the birth and early steps of the secretory pathway. Cargo synthesized in the ER continues through a series of dynamic compartments before reaching its final destination. This includes specialized sites on the ER called ER exit sites (ERESs), transport intermediates, and the Golgi apparatus. Here, we visualize ERESs using a cryo-correlative light electron microscopy (cryo-CLEM) system. Cells with ERESs fluorescently labeled by Sec23 were high pressure cryo-fixed, visualized by cryo-structured illumination microscopy (SIM), and then directly correlated to focused ion beam scanning electron microscopy (FIB-SEM). FIB-SEM data provides isotropic, high-resolution (4-8nm), large volume EM while cryo-SIM grants super-resolution (100nm lateral) protein specific localization. We found that ERESs were composed of a highly intertwined nest of tubules with a diameter of 360±60nm (n=110). The tubule diameter within the ERES was more constricted than the ER by 20%, 30±20nm (n=782). Using acargo synchronization and release system, RUSH (Retention Using Selective Hook), we directly studied cargo movement through the secretory pathway. When the ERESs were stressed by overloading with cargo we found that the total volume occupied by each ERES swelled 1.45-fold and the comprising tubule diameter expanded by 2.6-fold. Further investigation of coat protein localization at ERESs revealed that more than 70±10% (n=15 cells) of COPII positive ERESs also contained COPI. Using AiryScan microscopy and point localization techniques we observed a separation between COPI and COPII labeling on ERESs of 136±4nm (n=29), which increased by 1.45-fold with cargo overexpression. This was in agreement with FIB-SEM measurements of ERES sizes. Confocal imaging revealed that upon RUSH cargo release, COPI readily moved with cargo away from the ERES in transport intermediates, which often appeared as tubules, while COPII remained at the ERES. Applying cryo-CLEM in cells overexpressing RUSH cargo that were

cryo-fixed soon after cargo release allowed visualization of transport intermediates carrying RUSH cargo. Correlated FIB-SEM of these cells revealed that the transport intermediates existed as pearled membrane tubules running alongside microtubules. Among the pearled membrane tubules observed were those still connected to the ERES, those detached from ERESs, and those that had fused with the Golgi. Examples of the same structures were seen in FIB-SEM of untransfected cells. Together, this data provides a 3D view of the early secretory pathway never before seen with equivalent isotropic resolution, large depth-of-field, and precise protein localization.

P1326/B456

Mtorc1 Requires Ergic Vesicle Trafficking to Phosphorylate S6 Kinase, Not 4ebp1.

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The aim of this study is to characterize glutamine sensing by mTORC1 in RAS mutant cancers. In particular, the RAG GTPase independent glutamine sensing characterized by Jewell et al. was considered to describe a role for vesicle trafficking dependent mTORC1 activation in T24 and MDA-MB-231s (1,2). The study utilized protein knockdown, western blotting, and immunofluorescence assays to examine mTORC1 protein kinase activity under glutamine stimulation and vesicle trafficking inhibition at the ERGIC membrane compartment. The ERGIC-localized GEF GBF1 inhibiting drug Golgicide a (GCA) was utilized and characterized alongside rapamycin, a known mTORC1 inhibitor. The study's final conclusions are that by inhibiting ERGIC compartment vesicle trafficking, S6K phosphorylation by mTORC1 can be blocked entirely during glutamine sensing. Furthermore, by releasing the block on ERGIC derived vesicle trafficking 4EBP1 and GRB10 can be shown to be phosphorylated prior to S6K (and ULK1) and that S6K phosphorylation requires trafficking of the mTOR protein away from a Golgi-adjacent localization to one both on and in the nucleus, in both cell lines. Specifically, mTOR was found to saturate adjacent to the Cis and Trans Golgi membranes (but not on) during glutamine stimulation when GCA was included. Allowing the ERGIC compartment to reform and traffic mTOR shows a "backwards trafficking" event when tracked by the transmembrane ERGIC protein ERGIC53. Finally, the aforementioned information was utilized to "hyperactivate" P-S6K T389, meaning blocking and releasing ERGIC trafficking was used to increase S6K phosphorylation 400 percent above the normal glutamine stimulated increase. The sum of these results demonstrate that trafficking from the ERGIC compartment to both the Golgi and the ER are required for glutamine sensing in RAS mutant cancers via mTORC1. ERGIC trafficking manipulation can be used to uncouple mTORC1 target phosphorylation, and is particularly useful for determining the events and localization required of mTOR to phosphorylate S6K in RAS mutant cancers, some of which is characterized in this study. 1. Jewell, J.L., et al. (2015). Differential regulation of mTORC1 by leucine and glutamine. Science *347*, 194-198. 2. Xu, L., et al. (2011). Phospholipase D mediates nutrient input to mammalian target of rapamycin complex 1 (mTORC1). J Biol Chem *286*, 25477-25486.

P1327/B457

Cargo Crowding Drives Sorting Stringency in the Secretory Pathway.

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Accurate delivery of every protein to the correct compartment relies on both positive sorting signals and efficient retention of organelle resident proteins. In the endoplasmic reticulum (ER), secretory proteins are captured into transport vesicles by the COPII coat. These vesicles exclude ER resident proteins and

misfolded proteins by a mechanism that remains unresolved. Here we combined biochemistry and genetic methods with correlative light and electron microscopy (CLEM) to explore how this selectivity is achieved. We observed that changes in vesicle morphology do not fully explain the ER leakage phenotype in yeast mutants with defects in ER retention. Our data suggest that vesicle occupancy dictates ER retention in the context of these mutants: in the absence of abundant cargo, non-specific bulk flow increases. We demonstrate that ER leakage can be influenced by vesicle size and cargo occupancy. We propose that cargo recruitment into vesicles creates lumenal steric pressure that drives selectivity. Sorting stringency is thus an emergent property of the biophysical process of cargo enrichment into a constrained spherical membrane-bound carrier.

P1328/B458

Loss of the Short Form of Syntaxin-5 Causes Dysfunctional Golgi Trafficking and Glycosylation.

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The SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) protein syntaxin-5 is an essential Qa-SNARE for anterograde transport to the Golgi apparatus and intra-Golgi transport. In humans, the STX5 gene encodes two isoforms (Stx5 Short and Long) that result from alternative starting methionines at position 1 (Long) and 55 (Short). We identified a novel human disorder caused by a single nucleotide mutation in this gene, resulting in the loss of the short isoform (M55V). Patients carrying this mutation suffer severe pathologies, including abnormal N-glycosylation, skeletal disorders and very short survival (<3 months). In this study, we resolved the cellular mechanisms underlying these pathologies. Whereas Golgi morphology was unaltered in primary human dermal fibroblasts isolated from the patients, the cells showed defective glycosylation and mislocalization of glycosyltransferases MGAT1 and MAN2A1. Measurement of anterograde trafficking, based on biotin-synchronizable forms of Stx5 (the RUSH system) and determination of cognate binding SNAREs based on Förster resonance energy transfer (FRET) revealed functional differences of the long and short syntaxin-5 isoforms. Despite the presence of an ER-retention motif, the long isoform exits the Golgi faster and interacts more with early secretory pathway SNAREs, whereas the short isoform interacts more with intra-Golgi SNAREs. Thus, we identified a new trafficking disorder caused by mutations in a SNARE-encoding gene.

P1329/B459

Visualizing Procollagen and Hsp47 Trafficking in Live Cells.

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Currently, the mechanisms underlying ER to Golgi transport of large cargo are the subject of much controversy. For instance, there are three different hypotheses with regards to the trafficking of procollagen, which is the precursor of collagen, the most abundant protein in all vertebrates. Since the existing models are based on interpretation of mostly static imaging data, we performed high-speed (500 ms/frame), enhanced-resolution (~ 100 nm) dynamic imaging of procollagen transport vesicles in live cells. We developed multiple fluorescent protein-tagged constructs for transfection of immortalized osteoblastic cell lines (MC3T3). The dynamics of these fluorescent protein-tagged constructs were

visualized with time-lapse Airyscan imaging, including fluorescence recovery after photobleaching (FRAP) experiments. As procollagen-specific chaperone Hsp47 (Serpinh1) is commonly believed to be co-transported with procollagen from the ER to the Golgi, we imaged the dynamics of procollagen, HSP47, and fluorescent markers of different organellar compartments. We observed three-types of vesicle-like structures containing procollagen: (1) highly dynamic, rapidly moving procollagen transport vesicles (<500nm) exiting ER and entering Golgi that did not contain HSP47 and had no COPII coat; (2) static vesicle-like pools of dilated ER lumen that did contain HSP47; and (3) lysosomes that were relatively static and sometimes contained both procollagen and HSP47. These three vesicle-like compartments containing procollagen could be distinguished only by high-speed and high-resolution imaging with markers of ER lumen and lysosomes. Combined with previously published data, our observations indicate that procollagen transport vesicles emerge from ER exit sites (ERES) after ERES fusion with ERGIC membranes reduces pH and thereby releases HSP47 from procollagen. This interpretation is supported by the presence of the ERGIC membrane protein ERGIC53 in the transport vesicles.

P1330/B460

Isoform Specific IP3 Receptor Regulation of ER to Golgi Transport.

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Luminal calcium regulates vesicular trafficking from the ER to the Golgi. Previous studies indicate significantly reduced transport and a buildup of budding COPII vesicles and vesicle proteins upon severe luminal Ca^{2+} depletion. This may be due to Ca^{2+}-dependent COPII coat modulation at ER exit sites (ERES). However, the dynamics of Ca^{2+} involved in ER-to-Golgi transport are poorly understood. Inositol 1,4,5-trisphosphate Receptors (IP3Rs) release Ca^{2+} from the ER lumen upon agonist stimulation, triggering further Ca^{2+}-Induced Calcium Release (CICR) and hence, act as the nucleating step in ER Ca^{2+} signaling. Expression levels of the three isoforms of IP3R vary between cell types and biological differences between isoforms and homo- vs. hetero-tetramers are poorly understood. Using a single cell morphological assay for ER-to-Golgi transport in normal rat kidney (NRK) epithelia cells, we found that depletion of IP3R3, but not IP3R1, using siRNA resulted in significantly accelerated ER export of the membrane cargo VSV-G. Since NRK cells only express IP3R1 and IP3R3, this result implicated IP3R1, specifically IP3R1 homotetramers as playing a stimulatory role in ER export. Unexpectedly, live cell imaging using a FRET-based genetically encoded ER luminal Ca^{2+} sensor demonstrated a *decrease* in luminal Ca^{2+} concentration upon either IP3R1 or IP3R3 depletion. Imitating this change in luminal calcium with the Sarco/endoplasmic Reticulum Calcium-ATPase (SERCA) inhibitor Thapsigargin (TG), however, did not recapitulate the transport effects, indicating that the ER luminal Ca^{2+} concentration *per se* is not predictive of vesicular trafficking capacity. Rather, we suggest that IP3R1-regulated Ca^{2+} dynamics at ERES is a more rate-limiting determinant of secretion. Additional Ca^{2+} measurement experiments eliminated the possibility that the IP3R knockdowns altered basal ER leak or overall SERCA activity of the cells, further implicating Ca^{2+} signaling or other dynamics at ERES as the functionally critical parameter. In addition, the effects of IP3R knockdowns do not appear to be due to expression changes in COPII components and other trafficking machinery. We suggest that Ca^{2+} handling by IP3R1 at ERES controls a rate-limiting step in ER export.

9

Vesicle Docking and Fusion

P1331/B461

Sequestration of the Exocytic SNARE Psy1 into Multiprotein Nodes at Non-growing Regions of the Plasma Membrane.

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Spatial control of protein localization is important for many cellular processes in yeast and mammalian cells. Fission yeast is a long-standing model system for studies in cell morphology and polarity due to a highly polarized pattern of growth. These rod-shaped cells grow exclusively at their ends by restricting exocytosis and secretion to these sites. This growth pattern implies the existence of mechanisms that prevent exocytosis and growth along cell sides. We previously identified a set of 50-100 megadalton-sized node structures along the sides of fission yeast cells. On average, each node contains 70 copies of the interacting proteins Skb1 and Slf1. Here, we show that nodes also contain the syntaxin-like SNARE Psy1, which mediates exocytosis in fission yeast. Psy1 localizes throughout the plasma membrane with enrichment at cell tips, where it promotes exocytosis for polarized growth. At cell sides, where exocytosis should be prevented, Psy1 is sequestered in nodes with Skb1 and Slf1. Both Skb1 and Slf1 are necessary for Psy1 localization to nodes, and Psy1 associates with Skb1 and Slf1 by co-immunoprecipitation. Mutations that prevent node assembly lead to changes in the pattern of exocytosis and polarized growth, and these defects are exacerbated during osmotic stress. Studies suggest that spatial regulation of exocytosis is a common feature of most eukaryotic cells that is tightly linked to the overall polarity of a cell. Our work suggests that sequestration of syntaxin-like proteins at non-growing regions of the cell cortex reinforces cell morphology by restricting exocytosis to proper sites of polarized growth.

P1332/B462

Osmotic Squeezing and Membrane Tension Mechanically Drive Vesicle Evolution and Regulate Neurotransmitter and Hormone Release.

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Neurotransmitter and hormone release are fundamental to life and accomplished by exocytosis, when vesicles fuse with the plasma membrane (PM) to form omega-shaped membrane complexes that release contents via the fusion pore. It is widely accepted that the vesicle-PM complex evolves along one of two pathways, regulating contents release (Alabi and Tsien, 2013). (1) Full fusion. The pore dilates and the omega-profile flattens into the PM, with full contents release. (2) Kiss and run. The pore transiently opens and then closes, giving partial contents release. Recently this classical view was challenged by the Wu lab, using confocal and super-resolution STED microscopies to visualize neuropeptide release from dense-core vesicles in live chromaffin cells (Chiang et al., 2014; Wen et al., 2016). Instead of full fusion, they found full contents release is accomplished by the radically different “shrink-fusion” pathway, in which fused vesicles shrink into the PM retaining their Omega shape. Shrink fusion was suppressed by increased extracellular osmolarity or inhibition of F-actin by drugs or actin knockout (Wen et al., 2016). These findings are unexplained. Here we mathematically modeled the

evolution of dense core vesicles fused to the chromaffin cell PM and performed additional experiments. The model accounts for membrane bending energies and tension, osmotic forces and membrane-cytoskeleton adhesion, and is highly constrained by experiment. A novel mechanism emerged from the model, quantitatively explaining the experiments with no fitting parameters. Vesicle-PM merging is driven by: (i) osmotic squeezing, due to a reverse osmotic squeezing pressure on the vesicle, rapidly established following fusion, and (ii) membrane tension gradients that reel membrane from the vesicle onto the cytoskeleton, due to osmotic squeezing which deflates the vesicle and abolishes its tension, leaving the PM membrane tension unopposed. In agreement with experiment, a shrink-fusion sequence of vesicle shapes is predicted, separated by a large energy barrier (~140 k_BT) from the classical full collapse trajectory. Importantly, the model explains the observed dependence of Omega-shrink on the actin cortex (because actin-membrane adhesion helps reel in the vesicle membrane) and on extracellular osmolarity (which sets the osmotic squeezing pressure). The model predicts that sufficiently large vesicles are squeezed into elongated tubular shapes, a vivid manifestation of the novel osmotic squeezing mechanism. In new experiments we confirmed these remarkable tubular shapes with STED microcopy, strongly corroborating the squeezing forces. Comparing vesicle shapes from electron microscopy with shape predictions, we inferred that vesicles are squeezed with pressures from ~40 to ~400 Pa.

P1333/B463

Neurotransmitter Release Kinetics, Release Probability, and Calcium Sensitivity Are Regulated by Snare Proteins.

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Release of neurotransmitters (NTs) at neuronal synapses is evoked by calcium influx into the axon terminal of the presynaptic neuron. On sub-millisecond timescales, a remarkable multi-component machinery senses the calcium and fuses the synaptic vesicle and plasma membranes, releasing NTs through a fusion pore into the synaptic cleft. SNARE proteins constitute the core of the membrane-fusing component of the release machinery, but decades of studies assumed that NT release kinetics are principally regulated by the Ca-sensing component. How the two components of the machinery are coupled is poorly understood, and the possible role of the membrane-fusing component in regulating NT release has not been explored. In an important experimental study, NT release was enhanced in mouse Calyx of Held neurons by a constitutively open mutant of the t-SNARE Syntaxin which increased the number of assembled SNARE complexes at the fusion site (Acuna et al., 2014). The vesicle release probability, the NT release rate and the apparent Ca-sensitivity of release were all increased, while delay times and the apparent cooperativity of release were unaffected. Thus, NT release depends on the number of SNAREs and the ca-sensing and membrane-fusing components of the machinery appear coupled in a complex way. The molecular origin of these findings is unknown. Here, we present molecular dynamics simulations of the NT release machinery that realistically represent key molecular components for the first time. Our simulations use highly coarse-grained models of the neuronal SNARE complex (Mostafavi et al., 2017), and the Ca-sensor for synchronous NT release, Synaptotagmin 1. The model quantitatively reproduced three critical findings of the experimental study by Acuna et al. (1) We find that the reported acceleration of NT release and increased release probability is because the final membrane fusion step is faster when more SNARE complexes are present. The origin of the faster fusion is that more SNAREs generate stronger entropic forces that expand the SNARE complex ring at the fusion site and pull the vesicle and plasma membranes together with greater force. (2) the model

quantitatively reproduces the enhanced apparent Ca sensitivity of release observed in these experiments, and shows that it is due to increased probability that a binding event by a Ca ion unclamps a SNARE complex, i.e. releases it from a bound state with Synaptotagmin. (3) the model reproduces the finding that increasing the number of SNAREs does not affect the synaptic delay. This is because the most probable time for a vesicle to fuse with the plasma membrane is immediately following Ca-mediated unclamping of the SNAREs. Thus, synaptic delay primarily reflects ca-mediated unclamping, unaffected by the number of SNAREs present.

P1334/B464

The Neuronal Calcium Sensor Syt1 and Snare Proteins Cooperatively Dilate Fusion Pores in a Mechanical Lever Action.

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Neurotransmitters, hormones and other cargoes are released from vesicles through nanometer-sized fusion pores. Following fusion pore creation by SNARE proteins and auxiliaries, a vital step is the enlargement of the pore to allow contents release, but the mechanism of pore size regulation is unclear. Here we combined mathematical modeling and experiments to study the mechanisms. Conductance measurements of single fusion pores between nanodiscs and Hela cells showed that the calcium sensor Synaptotagmin-1 (Syt1) enlarges fusion pores in the presence of SNARE proteins and calcium. ~4 copies of full-length Syt1 and ~ 4 copies of VAMP2 per disc face were reconstituted into the nanolipoprotein particles. Compared with fusion pores in the presence of SNAREs alone, the mean conductance of fusion pores was three-fold larger in the presence of Syt1, calcium and SNAREs. We developed a mathematical model that quantitatively explained these findings, and suggests a novel mechanical post-fusion role for Syt1 during exocytosis. The Syt1 C2B domain is known to bind SNARE complexes, and the model incorporates Syt1-SNARE complexes following the published crystal structure (Zhou et al., 2015). We find a novel calcium-dependent post-fusion role for Syt1 in pore dilation, beyond its well-established pre-fusion function as the calcium sensor, in which Ca-triggered binding of the Syt1 C2 domains to the plasma membrane reorients the Syt-SNARE complex, tilting it upwards as a lever which pushes the fused membranes apart. This in turn enlarges the fusion pore due to the pore membrane energetics (with larger membrane separation, a larger pore has lower energy). In the model, SNARE complexes (SNAREpins) can be in the trans state (partially zippered) and free to roam (favored by entropy), or fully zippered at the waist of the fusion pore (favored by the zippering energy of the SNARE transmembrane domains). SNAREpins at the waist repel one another due to entropic crowding forces that expand the fusion pore, and can bind Syt C2B domains. The calcium-binding loops of C2B have a powerful tendency to bury into the target membrane when calcium is bound, an affinity which has been experimentally quantified and is incorporated into the model. The model predicts that the increase in membrane separation in response to calcium enlarges the pore radius from ~1 nm without calcium to ~1.7 nm, producing a ~2.3 fold increase in pore conductance, close to the experimentally measured ~2.2 fold increase.

P1335/B465

COG and GARP Vesicle Tethering Complexes Control the Fate of Golgi Glycosylation Enzymes.

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The Golgi is a central hub in the secretory pathway where cargo is delivered to undergo modifications and sorting to final destination. In order for Golgi's functions to be carried out properly, correct localization of the glycosylation enzymes within the Golgi sub-compartments is crucial. The eight subunit Conserved Oligomeric Golgi (COG) complex controls membrane trafficking and ensures Golgi homeostasis by orchestrating retrograde vesicle trafficking within the Golgi. In humans, COG defects lead to severe multi-systemic diseases known as COG-Congenital Disorders of Glycosylation (COG-CDG). The four subunit (VPS51-54) Golgi Associated Retrograde Protein (GARP) complex controls the retrograde transport from endosomes to TGN. The CRISPR approach was utilized to generate HEK293T cells deficient for individual COG and GARP subunits as well as for several double KO (DKO) combinations. COG KO cells show fragmentation of the Golgi cisternae, formation of the hybrid enlarged endolysosomal EELS compartment, defects in retrograde trafficking, sorting and glycosylation. GARP KO cells show defects in retrograde trafficking and, surprisingly, in both N- and O-glycosylation. Glycosylation and trafficking defects were even more severe in COG/GARP DKO cells. EELSs were mimicking some properties of late endosomes/lysosomes such as having an acidic lumen and endolysosomal membrane proteins, but lacking active lysosomal proteases. Lipid homeostasis was perturbed and some key Golgi lipids, including cholesterol and PI4P, were mislocalized to the EELS's membrane. Furthermore, tested Golgi resident proteins were found to undergo degradation in EELSs. Intriguingly, the maintenance of the EELSs was dependent on GARP activity showing interplay between the two complexes to regulate Golgi and endosomal homeostasis. Superresolution live cell microscopy, lectin staining, flow cytometry and EM were utilized to compare Golgi dynamics in KO mutants, wild-type and rescued cells. We found that stability of both cis/medial (MGAT1) and trans-Golgi (B4GALT1 and ST6GAL1) enzymes was compromised in COG and GARP KO cells, indicating that both tethering complexes are essential for the maintenance of Golgi glycosylation machinery. To gain a better understanding of Golgi enzyme fate in cells deficient for vesicle tethering complexes, we have used a RUSH pulse-chase approach, investigating intracellular trafficking and degradation of newly synthesized B4GalT1 and MAN2A1. We found that analyzed enzymes were not retained in the Golgi in both COG and GARP KO cells and, instead, were mistargeted to vesicles and endolysosomal degradative compartments. This work was supported by the NIH grant GM 083144

P1336/B466

The Three-dimensional Localization of Proteins on Single Exocytic Vesicles with Gold-labeled Fusion Proteins and Platinum-replica Electron Microscopy.

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Exocytic processes such as adrenaline and insulin release are regulated by a variety of proteins in eukaryotic cells. While the biogenesis, transport, and delivery of cargo-loaded vesicles (e.g. dense-core vesicles), and the proteins involved in these processes are well studied, how and where these proteins are organized at the nanoscale remains unknown. Here, we report a targetable genetically-encoded EM labeling method that uses a histidine-based affinity-tag system and metal-binding gold nanoparticles

along with 3D platinum replica transmission electron microscopy (PREM) and electron tomography to directly image proteins that are key regulators of exocytosis at the plasma membrane in mammalian cells. The method was validated by imaging well studied coat proteins on endocytic structures including clathrin-coated vesicles and caveolae. Next, the unknown 3D locations of key DCV associated proteins including Rab27a, Rabphilin, Rab3a, and Granuphilin were visualized on single vesicles in 3D at the scale of nanometers. The spatially averaged distribution of tagged gold particles revealed that these proteins are distributed across the entire surface of single docked vesicles. This global distribution of Rabs and Rab-binding proteins likely aids in the efficient capture and docking of DCVs in excitable cells. The nanoscale molecular architecture of DCVs generated from our method will help determine how key proteins assemble at the plasma membrane to regulate the docking, priming, and fusion of single DCVs in endocrine cells.

P1337/B467

Sphingolipids That Contain Very Long-chain Fatty Acids Regulate Homotypic Vacuole Fusion by Promoting the Vertex Enrichment of Regulatory Lipids, SNAREs, and Rab-/Rho-GTPases.

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Studies of sphingolipids in the endo-lysososmal membrane system of *Saccharomyces cerevisiae* are sparce due to the relatively low amounts found in these membranes. Here, we present evidence that sphingolipids containing very long-chain fatty acids (VLCFAs) positively regulate homotypic vacuolar fusion by acting as “regulatory lipids”. Vacuoles isolated from yeast cells that lack the C26 VLCFA elongase Elo3p display morphological abnormalities, and reduced amounts of in *vitro* fusion/hemifusion. We found that vacuoles isolated from C26 VLCFA deficient yeast fail to selectively concentrate a subset of SNAREs, Rab-GTPases, and other regulatory lipids at the vertex domain between apposed vacuoles. The vertex enrichment of regulatory lipids and fusion proteins on docked vacuoles is an interdependent process; they rely on each other for proper localization and timing of activity. These vacuoles were also found to lack Rho-GTPases, which impairs their ability stimulate actin polymerization. Studies using detergent resistant membranes isolated from wild type and mutant yeast vacuoles provide evidence that C26 VLCFA containing sphingolipids are a constituent of the vertex region, and they promote the clustering of fusion proteins. Taken together, these results suggest that sphingolipids containing C26 VLCFAs act as regulatory lipids in the homotypic vacuolar fusion cascade by promoting the protein/lipid assembly of vertex regions of docked vacuoles.

P1338/B468

Revealing the Mechanism That Controls Fusion Pore Dynamics in Giant Secretory Vesicles.

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Secretion by exocytosis occurs in every living cell and is essential for many cellular processes including metabolism, signaling and trafficking. To accommodate different cargos and cellular needs, exocytic vesicles are produced across multiple scales ranging from synaptic vesicles that are smaller than 50 nm in diameter to giant exocrine vesicles that reach up to a few μm in diameter. The later typically secret viscous cargos, such as digestive enzymes from pancreatic acinar cells and surfactant proteins from type II lung cells. Nevertheless, to enable cargo release these different types of vesicles all dock and fuse with

the plasma membrane. Yet, how vesicle size affects docking and fusion is poorly characterized on the mechanistic level. It is well established that during exocytosis of small vesicles, vesicle fusion can proceed in one of two morphological scenarios: in the first a fusion pore opens and expands irreversibly, leading to complete incorporation of the vesicular membrane to the cell membrane. The second is a "kiss-and-run" scenario, when the fusion pore "flickers", opening briefly and collapsing back, separating the two membranes. Here, we aimed to elucidate how fusion of giant vesicles progresses. We used the *D. Melanogaster* salivary gland, which secretes an adhesive protein through vesicles that are 5-8 µm in diameter, as a model. We visualized the secretion process using super resolution live-gland imaging and observed that unlike smaller vesicles, the fusion pore between giant vesicles and the membrane initially expand but then stabilize with a wide opening reaching up to 3 µm and subsequently constricting back down to hundreds of nm or less. Since constricting a membrane tube requires considerable energy input, we hypothesized that a dedicated protein machinery mediates this phenomenon. To identify this machinery we used the enormous power of Drosophila genetics to perform a candidate gene based screen and identified several conserved proteins from the BAR domain superfamily that act as key regulators of pore dynamics. This research has wide reaching implications on our understanding of exocrine secretion, leading to new and exciting insights into the molecular mechanism of membrane fusion and the regulation of membrane homeostasis in secreting cells.

P1339/B469

Membrane Homeostasis during Exocrine Secretion Is Maintained by Membrane Crumpling and Sequestration.

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Dynamic membrane trafficking comprises the essence of cellular homeostasis in every living eukaryotic cell. However, membrane dynamics becomes especially challenging in secretory epithelial tissues, where the continuous fusion of vesicles with the apical membrane takes place. This is most dramatically demonstrated in exocrine tissues that secrete viscous cargoes from giant vesicles, ranging up to 8 microns in diameter. Secretion of such giant vesicles adds large amounts of membrane to the apical surface of cells. Yet, it remains unclear how homeostasis of the cell surface in terms of size, shape, and composition is maintained under these extreme circumstances. To address this question, we used as a model system the *Drosophila* larval salivary gland, which secrets a viscous mucin-like protein called 'glue' via giant vesicles. After fusion with the apical membrane, a contractile actomyosin network is recruited to the surface of the vesicles to expel their content, and could thus be used as a marker to follow the fate of the vesicular membrane over time. Using live super-resolution microscopy, we observed that the vesicular membrane does not simply collapse into the apical surface during content release, but instead presents a crumpled appearance. To determine the precise ultrastructure of the vesicular membrane at different phases of secretion, we used correlative fluorescence and 3D-electron microscopy (CLEM). We observed that the vesicle membrane becomes increasingly crumpled and folded as secretion progresses, indicating that the membrane is not incorporated into the apical surface. To quantify this phenomenon, we used a correlative block-face and FIB-SEM approach and found that the compacted membrane indeed accounts for most of the original vesicular membrane before secretion. Furthermore, we found that the sequestered membrane recruits the clathrin-mediated endocytosis machinery, which recovers the membrane over a prolonged period of time post secretion. These results indicate that membrane homeostasis is maintained by actomyosin mediated crumpling and

sequestration of the vesicular membrane. This novel mechanism for maintaining membrane homeostasis may be ubiquitous and essential for exocrine tissue physiology.

P1340/B470

Human VPS33B and VPS16B Form a Multimeric Complex with a Two-lobed Structure.

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VPS33B and VPS16B are highly conserved proteins required for apical-basolateral cell polarization and vesicular trafficking in mammalian cells. Mutation of either *VPS33B* or *VPS16B* (*VIPAS39*) can cause ARC (arthrogryposis, renal dysfunction and cholestasis) syndrome, a neonatal lethal condition affecting multiple tissues. We have previously observed that VPS33B and VPS16B form a complex, VPS33B/VPS16B, that is required for the production of platelet secretory alpha granules by precursor megakaryocytes (*Blood* 2012;120:5032-40). VPS33B and VPS16B are homologous with the HOPS/CORVET complex components VPS33A and VPS16A, but these proteins and their complexes have distinct functions. While HOPS/CORVET has been well characterized, relatively little is known about VPS33B/VPS16B. We undertook to determine the composition and structure of VPS33B/VPS16B to gain insights into its role in megakaryocytes and other cells. Differential tag expression and blue native PAGE immunoblot analysis indicated the presence of at least 2 copies of each protein in the complex. VPS33B/VPS16B expressed in insect cells or isolated from human HEK293 cells co-migrated on native gels. A large-scale yeast expression and affinity purification system was developed to produce quantities of human VPS33B/VPS16B sufficient for biochemical analysis. Purified complex was analyzed via size exclusion chromatography with multi angle light scattering (SEC-MALS), small angle X-ray scattering, and single particle electron microscopy (EM). The results confirmed that each VPS33B/VPS16B complex contains multiple copies of both subunits. Low resolution envelopes obtained by negative staining EM and cryo-EM revealed a two-lobed VPS33B/VPS16B particle, with dimensions consistent with those of a predicted double heterodimer. Truncation of the VPS16B N-terminus did not affect subunit composition of the complex. We are currently examining the effects of amino acid alterations and VPS33B truncations on the size and structure of VPS33B/VPS16B.

P1341/B471

A Functional Genomics an alysis of 20 S Particle Proteins Involved in Plant Defense to Parasitic Nematodes.

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Prior RNA-seq-assisted analysis of a nematode-induced feeding structure in plant roots undergoing resistance resulted in the identification of alpha soluble NSF attachment protein (α-SNAP) whose induced expression resulted in suppressed levels of infection. The RNA-seq study also identified other genes expressed to high levels in these cells, but their role in resistance had not been tested. The genes include, several syntaxins (SYP), soluble N-ethylmaleimide-sensitive fusion factor adapter protein 25 (SNAP-25), N-ethylmaleimide-sensitive fusion protein (NSF), synaptobrevin (SYB), synaptotagmin (SYT), synaptosomal-associated protein 25 (SNAP-25) and vesicle associated membrane protein (VAMP). A

functional genomics analysis of some of these genes is providing a better understanding of how the proteins function in resistance.

P1342/B472

The Role of Munc13 in Snare Complex Formation.

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The role of Munc13 in SNARE complex formation Interneuronal communication through neurotransmitter release is crucial for brain function. Neurotransmitters are packed in synaptic vesicles and are released by calcium dependent fusion with the plasma membrane into the synaptic cleft where they trigger a signal on postsynaptic cells. There are many proteins involved in this process, but it is the SNARE complex that constitutes the core component of the vesicle fusion machinery. The SNARE complex consists of Synaptobrevin, Syntaxin-1 and SNAP25, which assemble into a tight complex through their SNARE motifs and bridge the membranes. Membrane fusion requires also other proteins that assist in forming and disassembling the complex. One of them is Mun13 which together with Munc18 is said to orchestrate the complex assembly. Munc18-1 closes Syntaxin-1 preventing it from forming the four helix bundle of the SNARE complex, but then binds to synaptobrevin to template assembly. Munc13 is believed to change the Syntaxin-1 conformation, stimulating the complex assembly. Although there is much data proving that Munc13 plays an important role in synaptic vesicle fusion, the exact mechanism remains unknown. To address this problem we are using a reconstitution approach, native gel assays and cross linking experiments. The data obtained so far supports the notion that Munc-13 modulates SNARE complex formation by opening Syntaxin-1.

P1343/B473

P4-Atpase TAT-1 Is Important for Phagosome-lysosome Fusion and Degradation of Residual Body.

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Cellular membranes are comprised of lipid bi-layers and membrane proteins. Distinct patterns of phospholipids with membrane proteins and their interactions can give rise to membrane system different identities, and can determine vesicles sorting and trafficking. Phosphatidylserine (PS) is one of the critical phospholipids, widely distributed in plasma membrane and endomembrane systems. In normal condition, PS is asymmetrically localized at the cytosolic side of lipid bi-layers, which is maintained by PS transporting enzymes. PS asymmetry is involved in apoptotic cell recognition and engulfment, and in endocytic trafficking processes. In worms, *tat-1* encodes a P4-ATPase, responsible for PS flipping. In *tat-1* mutant male gonad, residual bodies, generated during spermatogenesis, accumulate in the engulfing cell due to defects in clearance. Further experiments indicated that phagosome-lysosome fusion is delayed in *tat-1*, leading to residual body degradation defects. A retromer complex mutant was recovered in the *tat-1* suppressor screen. In *tat-1* mutant, retromer complex reporters stay longer on phagosome surface and hence postpone the fusion with lysosomes. During phagosome maturation, RabGTPase RAB-7 consecutively recruits retromer complex for cargo recycling and then HOPS complex for fusion. In *tat-1* mutant, we found that RAB-7 activity is higher, resulting in persistence of retromer complex on the phagosome surface and defects in phagosome-lysosome fusion.

P1344/B474

A Species-specific Mechanism Regulates Trimer Formation of Broadly Conserved Class Ii Fusogen Hap2 during Gamete Fusion in Chlamydomonas.

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The transmembrane protein HAP2 has been shown to be essential for gamete fusion in taxa across kingdoms. Although not yet detected in chordates and fungi, the distribution of HAP2 indicates that it is likely the ur-gamete fusogen. Its structural similarity to viral class II fusion proteins and its requirement by only one gamete of a fusing pair suggest that this eukaryotic fusogen will function similarly to its viral counterparts. The viral proteins undergo oligomeric rearrangements of their metastable pre-fusion forms into “hairpin”-like folded-back homo-trimers that drive merger of lipid bilayers during viral entry. Although the X-ray structures of HAP2 ectodomains showed a homo-trimer in the hairpin-like post-fusion conformation, this oligomeric state has not yet been detected in vivo. Thus, whether trimer formation occurs upon fusion in vivo and whether it is essential for gamete fusion remain unknown. Moreover, while low pH in the confines of target cell endosomes is the trigger for formation of trimers of class II fusion proteins in well-studied viruses (e. g., dengue and Zika), it is unlikely that HAP2 function will be controlled by a simple environmental cue. Here, we report that during gamete fusion in the unicellular green alga *Chlamydomonas reinhardtii*, HAP2 indeed forms oligomers with biochemical properties of homo-trimers, that trimer formation is essential for fusion, and that species-specific membrane adhesion is required for trimer formation. An alysis of HAP2 in unmixed mt- gametes by semi-native SDS-PAGE showed only monomers. As mt- gametes fused with mt+ gametes, pre-fusion HAP2 was converted into SDS-resistant trimers; and when fusion was complete, 90% of HAP2 remained in the pre-fusion form. Engineering mutations at the homo-trimer interface resulted in HAP2 mutants impaired in trimer formation in vitro and in vivo and incapable of supporting gamete fusion. Studies with fusion loop mutant forms of HAP2 impaired in their ability to insert into lipid bilayers indicated that bilayer insertion facilitated trimer formation. Furthermore, upon mixing with mt+ gametes, fusion loop HAP2 mutants incapable of gamete fusion formed unproductive trimers until most monomers were converted, indicating that trimer formation was regulated by fusion per se. Finally, fusion-driving trimers failed to form when mt- gametes were mixed with mutant *fus1* mt+ gametes lacking the species-specific membrane adhesion protein FUS1. Thus, broadly conserved eukaryotic class II fusogen HAP2 indeed forms trimers that drive gamete fusion, and formation of functional trimers depends on species-specific membrane adhesion. Supported by NIH R35 GM122565 to WJS, F32 GM126735 to JP, and F32 GM133158 to JZ. FR was supported by the European Research Council advanced grant Celcelfus.

P1345/B475

Functional an alysis of the ALPS Domain in *S. Cerevisiae* OSBP Homologue 4 (Osh4p).

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A wide range of cellular events require polarized exocytosis including, but not limited to, the formation of polarized intestinal epithelial cells and the formation of new daughter cells in yeast. In the budding yeast *S. cerevisiae*, the oxysterol binding protein homologue (Osh) family is required for polarized exocytosis and proper bud formation. In the absence of other Osh family members, Osh4p (Kes1p) is sufficient for polarized exocytosis, though in a manner that requires sterol and PI4P binding (Smindak *et*

al., *JCS* 2017). To date, many functional studies of Osh family proteins or mammalian OSBP-related proteins, both in *vitro* and in *vivo*, focused on lipid binding, modeling these proteins as lipid transfer proteins between closely apposed membranes. Structurally, these proteins have a lipid binding beta-barrel closed at one end and open at the other with a lid that can cover the open end of the barrel. The lid itself consists of the N-terminus of the protein; it can be either short, sufficient to cover the barrel, or long, presenting other functional domains in addition to covering the barrel. Although it appears that the lid controls protein orientation at membrane contact sites without affecting lipid transfer (Jamecna *et al*., *Dev Cell* 2019), less is known about the function of the lid in relation to specific cellular processes such as polarized exocytosis. In the case of Osh4p, the lid is short (29 amino acids) and consists of an Arf-GAP lipid packing sensor (ALPS) domain. Although it is known that the Osh4p ALPS domain influences sterol binding what, if any role, this protein domain has in Osh-dependent polarized exocytosis is unclear. We show that the Osh4p ALPS domain is essential for Osh4p function in polarized exocytosis, in particular exocytic vesicle docking at the plasma membrane (PM). We also show that the Osh4p ALPS domain decreases Osh4p localization to exocytic vesicles and sites of polarized exocytosis on the plasma membrane. Our results are consistent with a model in which the ALPS domain of Osh4p contributes to the temporal regulation of Osh4p.

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Neuronal Organelle and Protein Dynamics

P1346/B477

Structural Basis of GABARAP-mediated $GABA_A$ Receptor Trafficking and GABAergic Synaptic Transmission.

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$GABA_A$ receptor is one of the two major inhibitory receptors in the neuronal systems and responsible for balanced neural circuits. Dysfunction of trafficking and localization of $GABA_A$ receptor to the cell membrane is closely related to severe psychiatric disorders in humans. GABARAP serves as a main stabilizer for $GABA_A$ receptors localization on cell membranes, although the detailed molecular mechanisms are still not well understood. Here we show that GABARAP directly binds to a novel fragment of gamma2 subunit of $GABA_A$ receptor. The GABARAP/$GABA_A$ receptor crystal structure reveals the detailed interaction mechanisms governing the complex formation through a LIR-dependent manner. Overexpression of GABARAP in cells lead to $GABA_A$ receptor-mediated synaptic responses increase while mutations in either GABARAP or $GABA_A$ receptor blocking the binding dismiss the effect, in line with our in vivo electrophysiology results. We further showed GABARAP worked through the trafficking pathway rather than endocytosis in stabilizing GABA receptors. Our results correct the interaction between $GABA_A$ receptor and GABARAP and define a mechanism for synaptic localization of $GABA_A$ receptor through trafficking regulation by GABARAP.

P1347/B478

Functional an alysis Reveals Abnormal Expression of Gaba$_A$ Receptor Sub-units and Hypomotility Upon Loss of *Gabra1* in a Zebrafish.

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We used trio based whole exome sequencing (WES) to determine the genetic etiology of a patient diagnosed with a multi-system disorder characterized by a severe seizure disorder. WES identified a heterozygous *de novo* missense mutation in the *GABRA1* gene (c.875C>T), which resulted in a single amino acid substitution in one of the three transmembrane domains (p.Thr292Ile). *GABRA*1 encodes the alpha subunit of the Gamma-Aminobutyric Acid receptor a ($GABA_AR$). The $GABA_AR$ is a ligand gated ion channel that mediates the fast inhibitory signals of the nervous system and mutations in the sub-units that compose the $GABA_AR$ have been previously associated with human disease. To better understand the mechanisms by which mutations in *GABRA1* cause disease, we developed a zebrafish model of *gabra1* deficiency. *gabra1* expression is restricted to the nervous system and behavioral analysis of morpholino injected larvae suggests that the knockdown of *gabra1* causes hypoactivity. At a molecular level, hypoactivity is associated with defects in the expression of the most common sub-units of the $GABA_AR$. Collectively, our data raise the possibility that defects in *gabra1* expression, alter the normal composition of the $GABA_AR$, subsequently resulting in hypomotility.

P1348/B479

The Glutamate Receptor Glr-1 a Candidate Substrate for Endoplasmic Reticulum Associated Degradation in *Caenorhabditis Elegans*.

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Organisms require constant synthesis of proteins that are necessary to carry out specific functions at particular locations. For example, proteins found in cellular membranes are synthesized at the endoplasmic reticulum (ER). However, up to 30% of new proteins are improperly folded and must be removed. A build-up of misfolded proteins can trigger the Unfolded Protein Response (UPR) which initiates other pathways of protein quality control and determines the fate of a cell. ER-associated degradation (ERAD) is a ubiquitin-dependent process in eukaryotic cells that help alleviate protein accumulation by breaking down misfolded proteins recognized by the proteasome. The ERAD system is well described in yeast but is less well studied in multicellular systems. We use *C. elegans* as a model organism to study ERAD and a single membrane protein of interest that function in the pathway. Our membrane protein of interest is the glutamate receptor-1 (GLR-1), a model protein tagged with GFP (GLR-1::GFP), which allows us to observe if GLR-1 is a candidate substrate for ERAD. GLR-1 is expressed in a subset of interneurons in the ventral nerve cord (VNC) and accumulates at the synaptic membrane along the neurites of the VNC. We are assessing GLR-1's accumulation in the absence of ERAD E3 ubiquitin ligases in *C. elegans*. Three putative ERAD E3 ligases in *C. elegans* are: HRD-1, MARC-6, and HRDL-1. Deletion of most of the gene encoding HRDL-1, caused an increased accumulation of GLR-1::GFP in *C. elegans*. In addition, more of the accumulated protein (GLR-1) was retained at the ER in *hrdl-1* mutant animals, compared to controls. Together, these data suggest that *hrdl-1* has a role in regulating GLR-1 degradation at the ER and that GLR-1 may be a candidate endogenous substrate for ERAD. In order to understand how ERAD effects GLR-1, we are using tunicamycin to block ER-dependent glycosylation and initiate the UPR. Tunicamycin treatment induces ER stress, which activates a UPR-

reporter construct P*hsp-4*::GFP in *C. elegans*. An imals expressing the ER stress reporter *hsp4*::GFP respond to tunicamycin at lower doses when they harbor the mutation gene *hrdl-1*. We are using quantitative fluorescence imaging and immunoblotting to determine whether the GLR-1::GFP accumulation defects are due to underlying ER stress caused by the lack of HRDL-1, or if GLR-1::GFP's accumulation is due to a specific interaction between HRDL-1 on GLR-1::GFP.

P1349/B480

Understanding Clock Protein Dynamics and Localization Over the 24-hour Circadian Cycle.

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All living organisms including bacteria, plants, and mammals have evolved time keeping mechanisms such as circadian clocks to synchronize their internal state to the external environment. Almost every cell in the human body has a molecular clock, which is based on a negative transcription-translation feedback loop, and these clocks orchestrate daily rhythms in many physiological processes, from sleep to metabolism. In our studies we use the *Drosophila melanogaster* clock neurons as they feature a highly conserved clock similar to humans and are amenable to powerful genetic and molecular tools. Specifically, the *Drosophila* clock is based on a negative transcription-translation feedback loop made up of four key clock genes–Clock (CLK), Cycle (CYC), Period (PER), and Timeless (TIM). In preliminary studies we performed 4-D live-cell super-resolution imaging to investigate the subcellular spatio-temporal dynamics of PER in individual clock neurons in *Drosophila* brains over the 24-hour cycle. Specifically, we generated fluorescent protein tagged flies (endogenous PER tagged with mNeonGreen, a green fluorescent protein) using CRISPR and first confirmed that the tagged protein is functional. Next, from live imaging studies we found that PER is concentrated in distinct, dynamic nuclear foci (*i.e.* change in position and size with time) in the clock neurons—through a process of liquid-liquid phase separation. We are currently investigating the role of clock protein phase separation in the regulation of circadian clocks. This work is expected to provide important new insights into how circadian rhythms are regulated and also illuminate general principles of gene regulation and phase separation in cellular organization.

P1350/B481

The Glycolytic Protein Phosphofructokinase Dynamically Relocalizes into Subcellular Compartments with Liquid-like Properties in *Vivo*.

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While much is known about the biochemical regulation of glycolytic enzymes, less is understood about how they are organized inside cells. Here we built a hybrid microfluidic-hydrogel device for use in *Caenorhabditis elegans* to systematically examine and quantify the dynamic subcellular localization of the rate-limiting enzyme of glycolysis, phosphofructokinase-1/PFK-1.1. We determine that endogenous PFK-1.1 localizes to distinct, tissue-specific subcellular compartments in *vivo*. In neurons, PFK-1.1 is

diffusely localized in the cytosol, but capable of dynamically forming phase-separated condensates near synapses in response to energy stress from transient hypoxia. Restoring animals to normoxic conditions results in the dispersion of PFK-1.1 in the cytosol, indicating that PFK-1.1 reversibly organizes into biomolecular condensates in response to cues within the cellular environment. PFK-1.1 condensates exhibit liquid-like properties, including spheroid shapes due to surface tension, fluidity due to deformations, and fast internal molecular rearrangements. Prolonged conditions of energy stress during sustained hypoxia alter the biophysical properties of PFK-1.1 in *vivo*, affecting its viscosity and mobility within phase-separated condensates. PFK-1.1's ability to form tetramers is critical for its capacity to form condensates in *vivo*, and heterologous self-association domain such as cryptochrome 2 (*CRY2)* is sufficient to constitutively induce the formation of PFK-1.1 condensates. PFK-1.1 condensates do not correspond to stress granules and might represent novel metabolic subcompartments. Our studies indicate that glycolytic protein PFK-1.1 can dynamically compartmentalize in *vivo* to specific subcellular compartments in response to acute energy stress via multivalency as phase-separated condensates.

P1351/B482

Regulation of Mbp Local Translation Is Crucial for Adult Myelin Maintenance.

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Efficient electrical signaling along axons depends on formation of compact myelin. This process of compaction requires myelin basic protein (MBP) to function as a molecular zipper to exclude cytoplasmic proteins and organelles from regions of compact myelin. *Mbp* is the most highly expressed mRNA in oligodendrocytes by 10-fold and it is transported along oligodendrocyte processes by kinesin and dynein before it is locally translated. Here, we further elucidate the mechanisms that regulate MBP local translation. First, ribosomal abundance inversely correlates with MBP protein levels. In primary oligodendrocytes, ribosomes accumulate slowly on the timescale of days prior to MBP protein translation. However, oligodendrocytes with high levels of MBP protein have few ribosomes, indicating that ribosomes may be degraded following MBP translation. In compartmentalized Boyden chambers, RNA isolated from oligodendrocyte processes contain high levels of ribosomal RNA, indicating that ribosomes may themselves be locally translated. Second, we ask whether transport of *Mbp* mRNA is required for MBP translation. In a proteomic screen using the RNA-binding reporter MS2, we found that *Mbp* mRNA associates with a myosin motor that is mutated in patients with distal myopathy and white matter lesions. We show that the myosin inhibitor blebbistatin causes *Mbp* mRNA to accumulate along microtubules, no longer distribute along actin, and also blocks MBP translation. We confirm this effect is specific to *Mbp* mRNA using a mouse model that replaces the *Mbp* 3'UTR with a stabilizing polyA sequence; these mice still express the *Mbp* CDS and thus are theoretically capable of translating MBP. Oligodendrocytes cultured from these mice strikingly lack *Mbp* mRNA granules outside the cell body, but have aberrant, large donut-shaped *Mbp* mRNAs outside the cell body that co-localize with late endosomes/lysosomes markers (Rab7, LAMP1). However, in both 2D and 3D microfiber cultures, these cells do translate MBP outside of the cell body. Thus, though 3'UTR-less *Mbp* mRNA can nonspecifically localize outside the cell body, they cannot be translated. This is consistent with the striking phenotypes of these adult mice - hypomyelination, tremors, and severe motor coordination defects. Third, we measure the dynamics of MBP translation. Using a photoactivatable reporter, we find that MBP translation does not occur gradually, but mostly in the fifth day of differentiation. Imaging with membrane-anchored GFP, we observe compaction events that occur rapidly, on the timescale of 3-5 minutes. Together, these experiments indicate that MBP translation is a highly regulated event that

relies on the convergence of ribosome accumulation and regulation of *Mbp* mRNA transport and translation activation via the 3'UTR.

P1352/B483

Dynamics of Endoplasmic Reticulum Proteins in Nerve Terminals.

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The endoplasmic reticulum (ER) is the largest membrane-enclosed organelle in eukaryotic cells. It spreads throughout the cytoplasm, where it plays an essential role in membrane and secretory protein synthesis, in the biogenesis of most membrane lipids, Ca^{2+} storage and metabolite processing. The ER is a network of interconnected tubules and cisternae that forms specialized subdomains such as the nuclear envelope, the ribosome-rich ER (RER) and the smooth ER (SER). The three-dimensional reconstruction of subcellular organelles from FIB-SEM image stacks of mouse brain tissue revealed that neuronal cell bodies and dendrites are enriched with a continuous network of tubules and cisternae decorated by ribosomes[1]. In axons small narrow tubules of smooth ER are most abundant[2], and thin axonal segments contain a single continuous tubule of smooth ER that branches or expands in small cisternae at synaptic varicosities[1]. Several mutations in ER-resident proteins have been reported to cause the neurological disorder hereditary spastic paraplegia[3], however the role of axonal ER at the nerve terminal is still not well understood. It has been recently shown that changes in axonal ER luminal calcium regulate nerve terminal function through a mechanism involving the ER membrane protein STIM1. When ER calcium levels drop significantly below ~ 150 µM, STIM1 is mobilized and inhibits presynaptic function, through a poorly characterized mechanism [4]. In order to gain insight into the dynamics of ER proteins in axons we have used single particle tracking of Halo-tagged ER proteins, including STIM1. Given the highly dynamic rearrangements or ER structures and the rapid redistribution or ER proteins[5,6], we hope to elucidate its role in the modulation of presynaptic function.

P1353/B484

A Novel System for Compartmentalized Study of Neuronal Intermediate Filament Proteostasis in Axons.

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Sensory and motor axons extend long distances to transmit electrical impulses between the central nervous system and the periphery. Neuronal intermediate filaments (IFs), which include neurofilaments, α-internexin and peripherin, are major structural components of the axon. Several neurodegenerative diseases present with large axonal swellings that are sites of localized IF protein accumulation. Axonal IF protein accumulation directly impairs neuronal function, which is most dramatically exemplified in the progressive and fatal neurodegenerative disorder giant axonal neuropathy (GAN). GAN is caused by loss-of-function genetic mutations in the E3 ubiquitin ligase adaptor protein gigaxonin, which controls IF protein turnover. This work aims to develop novel patient-derived cellular models to study and target axonal dysfunction caused by IF protein accumulation in GAN. We generated induced pluripotent stem cells (iPSCs) by reprogramming skin fibroblasts from seven GAN patients carrying gigaxonin mutations the severity of which is associated with specific motor function measure scores (MFM). We used CRISPR/Cas9 gene editing to correct the homozygous missense mutation p.G332R in the iPSCs line

derived from a patient with severe symptoms, as determined by the MFM score (30.2/100). Using established motor neuron differentiation protocols, we differentiated the iPSCs to neural progenitor cells (NPCs) and seeded them on pre-assembled microfluidic devices (XonaChipTM). The NPCs were differentiated to motor neurons using defined media conditions for 45 days. We used confocal microscopy to investigate the expression, localization, and aggregation of several IFs proteins at cell soma, dendrites, and along the axons in both the isogenic controls and parental cells carrying the disease mutations. The use of microfluidic system, coupled with confocal imaging, dramatically improve the localization analysis and resolution of IF protein aggregates along the length of the axons. This system revealed abundant aggregation of neurofilaments (heavy and medium), phospho-neurofilament, and α-internexin. The imaging analysis further revealed differences in aggregate size and distribution pattern along the length of the axon, likely reflecting differences in protein composition of the inclusions. In contrast, isogenic control iPSC-derived motor neurons displayed normal filament structures with smooth and uniform axons. In conclusion, we successfully established a novel in vitro tool to study defective IF proteostasis in axons within a microenvironment distal to the cell soma. This system enables investigation of GAN disease mechanisms and testing of therapeutic candidate compounds to alleviate IF protein accumulation specifically in axons.

P1354/B485

Direct Delivery Is the Predominant Trafficking Pathway of Axonally Polarized Membrane Proteins.

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Neurons are polarized cells with axons and dendrites. These domains serve different functions in electrochemical signaling and require different complements of plasma membrane proteins. Membrane proteins are synthesized in the cell body and delivered to neurites via microtubule-based vesicle transport. Because axons can be up to a meter long in humans, they are particularly reliant on vesicle transport. The exact trafficking pathway of axonal membrane proteins is not well understood. Two models have been proposed: 1) in the direct delivery model, proteins are packaged into axon-selective vesicles at the Golgi. These vesicles can move in dendrites but only fuse with the axonal plasma membrane. 2) in the transcytotic model, proteins are packaged into vesicles that fuse with the dendritic plasma membrane. There they are endocytosed into vesicles that fuse with the axonal plasma membrane. The relative contribution of these two pathways towards the delivery of axonal membrane proteins is unknown and has been a long-standing question in neuronal cell biology. Because different sorting machineries act at the Golgi and the plasma membrane, determining the location of protein sorting into axon-selective vesicles is a crucial step towards understanding how axons are maintained. We developed a novel assay in hippocampal neurons to determine if any given axonal vesicle contains transcytotic proteins. We engineered modified versions of two important axonal proteins, neuron-glia cell adhesion molecule (NgCAM) and vesicle-associated membrane protein (VAMP) 2. We designed these proteins with a streptavidin-binding peptide in their ectodomain and a GFP label. Fluorescent streptavidin in the culture medium only labels vesicles that contain endocytosed proteins. Transcytotic vesicles are labelled with both GFP and streptavidin while vesicles directly delivered to the axon are only labeled with GFP. Two-color live-imaging of axonal vesicles found that the vast majority of axonal NgCAM vesicles (>95 %) and nearly three quarters of axonal VAMP2 vesicles (72 %) were the product of direct delivery. Simultaneous labeling of NgCAM and VAMP2 found that both proteins occupied the same Golgi-derived vesicles, whereas transcytotic VAMP2 vesicles were a distinctly different population.

These results have important implications for understanding neuronal membrane trafficking. Direct delivery is the predominant sorting pathway for axonally polarized membrane proteins. A small population of VAMP2 arrives in the axon via transcytosis, suggesting that this protein interacts with two different sorting machineries. The sorting machineries and sorting signals that target axonal membrane proteins are as yet undetermined.

P1355/B486

A Novel Labeling Strategy Reveals That Neuronal Myosin V-labeled Vesicles Are Polarized to Dendrites.

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The maintenance of the eukaryotic endomembrane system requires accurate trafficking of proteins between different cellular compartments by vesicle transport. This is particularly important for neurons, which have numerous subdomains that require specific complements of membrane proteins. Kinesins and dynein mediate long-range microtubule-based transport, while myosins mediate short-range actin-based transport. Myosin V is the primary processive motor that mediates transport towards the barbed end of actin, but little is known about its neuronal functions and its interactions with other motors. Identification of Myosin V cargoes is crucial for determining its neuronal function. One obvious approach to address this is to coexpress fluorescent Myosin V and candidate cargo proteins in the same cell. This is challenging because overexpression of full-length Myosin V results in a bright soluble pool that masks labeled vesicles. We recently developed a strategy to visualize vesicle-bound kinesins by expressing their vesicle-binding tail domains. Here we applied this approach to Myosin V in hippocampal neurons. Live-cell imaging found that Myosin V-labeled vesicles were polarized to the somatodendritic domain and many of these vesicles underwent long-range transport. To determine the identity of these vesicles, we coexpressed Myosin-V tail and important neuronal vesicle trafficking proteins. We found that Myosin V co-transported with the dendritically polarized transferrin receptor, vesicles associated with Kinesin-3 family motors KIF13A and KIF13B. Myosin V also co-transported with a subset of vesicles carrying the dendritically polarized low-density lipoprotein receptor, vesicles associated with the Kinesin-3 motors KIF1A and KIF13B. Unpolarized dense-core granules labeled with brain-derived neurotrophic factor and axonally polarized vesicles labeled with neuron-glia cell adhesion molecule did not colocalize with Myosin V. We systematically measured overlap between Myosin V vesicles and important neuronal cargo proteins. Our results suggest that Myosin V binds to vesicles concurrently with several Kinesin-3 family members. This raises an important question: How are Myosin V and kinesin activity coordinated on the same vesicle? Our findings are also consistent with a previously proposed role for Myosin V in mediating retention of dendritically polarized vesicles in the proximal axon. The novel labeling strategy vastly enhances visualization of Myosin V on vesicles and should be broadly applicable for investigating Myosin V function in many cell types.

P1356/B487

Endocytosis of Dendritic Membrane Proteins in the Axon Initial Segment Is Essential for Neuronal Compartmentalization.

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How membrane proteins are differentially trafficked to specific domains within a cell is a long-standing question in cell biology. Due to their large size and extreme compartmentalization into axons and dendrites, neurons especially rely on divergent membrane trafficking mechanisms. The axon initial segment (AIS) of the neuron is a specialized boundary zone between the axon and dendrite, and it acts as a selective filter and/or diffusion barrier to achieve protein compartmentalization. We delineate a novel active sorting mechanism in the AIS in which dendritic membrane proteins are endocytosed and degraded to prevent their entry into the axon. We identified this mechanism by studying the dendritic morphology guidance receptor, DMA-1, which is required for the development of the *C. elegans* PVD neuron dendrite, but not its axon. Endogenously labeled DMA-1::GFP is highly polarized to the PVD dendrite and excluded from the axon. We found that DMA-1 undergoes endocytosis in the AIS and localizes to late endosomes. Endogenously labeled clathrin light chain localizes to multiple puncta in the AIS, a region not previously recognized as a site of endocytosis. Together, these results suggest that DMA-1 endocytosis in the AIS is an active removal mechanism to prevent axon entry. Indeed, we found that endocytic inhibition using a temperature sensitive dynamin mutant causes DMA-1 mislocalization to the axon and aberrant axonal branching. We also identify a novel DMA-1 interacting protein called LRP-1 like (LRPL-1), which is essential for DMA-1 endocytosis in the AIS. Therefore, we have delineated a novel mechanism in which the AIS dictates the membrane trafficking and localization of neuronal receptors. Inhibition of this active endocytic sorting mechanism results in the loss of protein compartmentalization and alteration of neuron morphology. These results define a new framework for understanding membrane trafficking mechanisms in the axon initial segment of the neuron.

P1357/B488

Activity Induces Presynaptic Mitochondria Capture and Energy Maintenance Via Syntaphilin-mediated an choring and Ampk Energy Sensing Pathway.

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Mitochondria supply ATP essential for supporting high energy-demanding synaptic transmission. Due to their unique polarized structure, neurons face exceptional challenges in maintaining presynaptic energy supply by delivering and anchoring mitochondria at distal synapses. The loss of presynaptic mitochondria inhibits synaptic transmission. Synapses are highly plastic and undergo activity-dependent remodeling; thereby constantly changing mitochondrial re-distribution to meet dynamic energy consumption. Interestingly, sustained synaptic activity is mainly restricted to these mitochondria-containing presynapses during long-term potentiation. Presynaptic mitochondria can be re-mobilized and re-distributed in response to changes in synaptic activity (Sheng 2014). However, the mechanisms recruiting and capturing axonal mitochondria at presynapses in response to changes in synaptic activity and local energy consumption remain largely unknown. Here, we reveal the new energy signaling cascade that controls activity-dependent presynaptic mitochondrial capture and energy maintenance. Syntaphilin plays a crucial role in recruiting and capturing axonal mitochondria at presynaptic terminals

via cytoskeletal track-switch from axonal microtubules to actin filaments that is enriched at synapses. Sustained synaptic activity and thus high ATP consumption activate AMPK signaling, which facilitates mitochondrial recruitment through phosphorylation of downstream anchoring machinery. This pathway allows neurons maintain presynaptic energy supply essential for prolonged and repetitive synaptic transmission. Inhibiting AMPK energy signaling or interfering with the anchoring machinery reduces presynaptic ATP availability, leading to synaptic depression after prolonged stimulation. Thus, our study provides new mechanistic insights into action of cellular energy sensing pathway in the maintenance of presynaptic ATP availability by recruiting axonal mitochondria in response to increased synaptic activity (Supported by the Intramural Program of NINDS, NIH). Reference: Sheng ZH (2014). Mitochondrial trafficking and anchoring in neurons: New insight and implications. J Cell Biol. 204(7):1087-1098.

P1358/B489

Suppression of Axonal Transport of Mitochondria Disrupts Protein Degradation Pathways and Induces Accumulation of Ubiquitinated Proteins in the *Drosophila* Brain.

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Mitochondrial transport in the neuronal axon plays critical roles in physiology and pathophysiology. Defects in axonal transport of mitochondria have been suggested in the pathogenesis of a number of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Abnormal accumulation of proteins in neurons are another pathological feature of these diseases and thought to contribute to neuronal death. These observations indicate that disruption of protein homeostasis and intracellular distribution of mitochondria are both play critical roles in neurodegeneration in these disorders. However, the role of mitochondria in protein homeostasis is not well understood. Here we report that disruption of mitochondrial distribution to the axon causes an accumulation of ubiquitinated proteins in the brain in *Drosophila*. Neuronal knockdown of milton or Miro, an adaptor protein for axonal transport of mitochondria, causes depletion of mitochondria from the axon and causes age-dependent neurodegeneration. To ask whether proteostasis is affected in these fly brains, we analyzed the levels of ubiquitinated proteins at different ages. Western blot and immunostaining revealed that milton knockdown causes accumulation of ubiquitinated proteins in young fly brains before the onset of neurodegeneration. Ultrastructural analysis of the photoreceptor neurons with milton knock down detected dense material suggesting protein accumulation. We further analyzed whether protein degradation pathways were disrupted by milton knockdown. Western blot of autophagy markers LC3-I/II and p62 as well as analyses of autophagic fluorescent reporter revealed that autophagic flux is reduced in fly brains with milton knockdown. Finally, to gain insight into the underlying mechanism, we tested whether an overall reduction in mitochondrial functions was sufficient to cause accumulation of ubiquitinated proteins in the brain. We found that overall reduction in mitochondrial function by mutation in mitochondrial ribosomal protein S12 did not cause either accumulation of ubiquitinated proteins or autophagic deficits. These results suggest that accumulation of ubiquitinated proteins observed in milton knockdown flies are due to mislocation of mitochondria rather than a reduction in

mitochondrial function in the axon. Our results suggest that the reduction in the axonal distribution of mitochondria causes disruption in proteostasis and may contribute to the onset and progression of neurodegenerative diseases.

P1359/B490

Four and a Half LIM Domains Protein 2 Mediates Mitochondrial an choring to F-actin.

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Mitochondria form distinct pools of stationary and motile organelles. The movement of the motile pool of mitochondria is powered by microtubule based molecular motors. The motors are recruited to the mitochondria by a complex of two proteins on the outer mitochondrial membrane: Miro (also called RHOT 1/2) and Milton (also called TRAK 1/2). Here we report a mechanism by which the Miro/Milton complex, and thereby the mitochondria, are anchored to the F-actin cytoskeleton in response to changes in glucose availability. The immobilized mitochondria retain their molecular motors and can regain their motility when the actin network is disrupted. This pathway of mitochondrial anchoring to F-actin is activated by an acute influx of glucose. This influx triggers the O-GlcNAcylation of Milton by the enzyme O-GlcNAc transferase (OGT). The O-GlcNAcylated Milton binds the actin-binding protein, Four and a half LIM domains protein 2 (FHL2), and thus becomes anchored to the F-actin cytoskeleton. To support this model we demonstrate that: 1) a glucose influx or the over-expression of OGT leads to O-GlcNAcylation of Milton and immobilizes mitochondria; 2) F-actin is responsible for immobilizing the O-GlcNAcylated mitochondria; 3) FHL2 co-immunoprecipitates with Milton under conditions that promote its O-GlcNAcylation; 4) mutation of the O-GlcNAcylation sites on Milton prevents its association with FHL2; 5) knockdown of FHL2 prevents mitochondrial arrest even in the presence of O-GlcNAcylated Milton; 6) artificially targeting FHL2 to the outer mitochondrial membrane is sufficient to drive the association of mitochondria with actin and to stop their movement. Therefore, the recruitment of FHL2 to the mitochondrial surface upon Milton O-GlcNAcylation is both necessary and sufficient to arrest mitochondrial movement by tethering to F-actin. In non-neuronal cells, FHL2 mediated mitochondrial anchoring results in a significant increase of F-actin density around mitochondria. In axons, however, the mitochondria appear to anchor to preexisting actin filaments without any cytoskeletal rearrangement. This mechanism of mitochondrial anchoring to F-actin likely operates in *vivo*. We have previously observed that Milton O-GlcNAcylations are altered by a fasting/feeding protocol (Pekkurnaz et al., 2014). Further, we find that changes in extracellular glucose concentrations, which mimic serum glucose fluctuations post feeding, arrests mitochondria in an F-actin and FHL2 dependent manner. This phenomenon of O-GlcNAcylation mediated mitochondrial arrest appears to be pervasive across multiple cell types and across different species, from *Drosophila to* mammals. Thus, the mechanism is likely conserved and broadly utilized for adapting mitochondrial dynamics to local glucose supply.

P1360/B491

RETROGRADE Transport Is Required for Mitochondrial Health and Function in Neurons.

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Maintenance of functional neural circuits is critical for organism survival. To form and maintain the elaborate structure of a neuron, proteins and organelles must be transported to the correct location

throughout the cell. One organelle of particular importance is mitochondria. Mitochondria produce ATP and regulate cytosolic calcium, among perhaps lesser known functions, critical for neuronal maintenance. Abnormalities in mitochondrial transport are associated with neurodegenerative diseases but the mechanisms leading to pathology are largely unknown. While we have a basic understanding of the molecular regulators of anterograde transport of mitochondria (axon terminal directed), the mechanisms regulating retrograde (cell body directed) transport and the actual function of this process were not clear. Furthermore, the dynamics of mitochondrial movement in neurons on timescales longer than minutes were completely unknown. To begin to address these long-standing questions, we used photoconversion and long-term tracking of mitochondria in vivo to assess the frequency of retrograde mitochondrial transport. Our results indicate that mitochondria in mature axons are largely stationary on the order of minutes but move long distances over the course of hours. Strikingly, mitochondria that originate in axon terminals utilize retrograde transport to return to the cell body from the distal axon with complete population turnover from axon terminals within three hours. Our data also illustrates that this process is not just for mitochondrial degradation: inhibition of retrograde mitochondrial movement leads to loss of cell body mitochondrial load. Using various fluorescent reporters, we found that organelles which cannot move back to the cell body show an imbalance of the oxidation/redox system, loss of matrix potential and impaired calcium buffering capacity in neurons. Finally, we provide evidence that retrograde mitochondrial transport back to the cell body is important for maintaining the mitochondrial proteome, likely by bringing the organelle back to the center of protein synthesis for the cell. Altogether, our work has shown the first direct evidence that retrograde transport is essential to maintain mitochondrial homeostasis in neurons.

P1361/B492

Als-associated Mutations in Tbk1 Differentially Disrupt Mitophagy Kinetics.

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TANK-binding kinase-1 (TBK1) has been implicated in several key cellular pathways, including the clearance of damaged mitochondria via mitophagy. The wild-type TBK1 molecule functions as a dimeric kinase that is activated by trans-autophosphorylation to stabilize components of the mitophagy machinery and promote clearance of the damaged organelle. Several mutations of TBK1 are causative for the neurodegenerative disease amyotrophic lateral sclerosis (ALS), but their respective effects on the mitophagic mechanisms are unknown. Interestingly, these mutations occur throughout the structure of TBK1, and are predicted to disrupt a variety of its functions, including kinase activity, dimerization, and overall stability of the molecule. We complemented structural and biochemical analyses of TBK1 mutants with fluorescence microscopy to test whether each mutant could form mitophagy rings after mitochondrial damage. A monomeric TBK1 mutant, R357Q, forms rings around damaged mitochondria. However, TBK1 mutants with abolished auto-phosphorylation activity, G217R (dimeric) and M559R (monomeric), demonstrate significantly less ring formation. Meanwhile, R47H, a mutant with weak autophosphorylation activity does form mitophagy rings, but has poor co-recruitment of TBK1's mitophagy partner, optineurin (OPTN). With live cell timelapse imaging, we can dissect the kinetics of mitophagy components throughout the pathway and compare the findings among TBK1 mutants. M559R never translocates to damaged mitochondria, while G217R can be weakly recruited. Together, these results indicate that the various ALS-associated mutations of TBK1 disrupt mitophagy at a range of

steps, which may be relevant in developing targeted therapies. This project is supported by Project ALS and R37 NS060698.

P1362/B493

Misregulation of Mitochondria - Lysosome Contact Sites in Mutant GBA Parkinson's Patient Neurons.

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Parkinson's disease (PD) is the second most common neurodegenerative disorder and has been linked to defects in both mitochondrial and lysosomal function. However, while mitochondria and lysosomes are critical organelles for maintaining neuronal homeostasis, how their dynamics are bidirectionally regulated in neurons is still not well understood. Our lab recently identified the dynamic formation of inter-organelle mitochondria-lysosome contact sites in non-neuronal cells which are essential for regulating the network dynamics of both mitochondria and lysosomes (*Wong et al., Nature 2018*). However, the role of mitochondria-lysosome contact sites in human neurons, as well as their contribution to Parkinson's disease pathogenesis has not been previously studied. Using human iPSC-derived dopaminergic neurons, we found that mitochondria-lysosome contact sites dynamically form in the cell body, axon and dendrites of human neurons. Interestingly, mitochondria-lysosome contact sites are potentially affected by lysosomal enzymes, as reduced activity of the lysosomal enzyme GBA (β-glucocerebrosidase) disrupts lysosomal dynamics contributing to defective mitochondria-lysosome contact site formation. Moreover, heterozygous GBA mutations in familial Parkinson's disease patient neurons exhibit dysfunctional mitochondria-lysosome contact dynamics, resulting in disrupted mitochondrial dynamics in both the axon and cell body. Together, these findings may advance our understanding of fundamental biology underlying the interplay between mitochondria and lysosomes in neurons, and provide important insights into disease pathogenesis in Parkinson's disease.

P1363/B494

Impaired Lysosome Transport to Distal Axons Contributes to Autophagic Stress in the Neurodegenerative Lysosomal Storage Disorder Niemann-Pick Type C.

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Niemann-Pick Type C (NPC) is a neurodegenerative lysosomal storage disorder characterized by accumulation of multiple lipids in late endosomes and lysosomes. An early pathologic feature of NPC is axonal dystrophy, which consists of bulbous swellings along axons that contain accumulated organelles associated with the autophagy-lysosomal pathway. Such changes occur before symptom-onset and neurodegeneration in NPC mice and suggest that defects in axonal organelle transport contribute to early NPC pathology. However, the mechanisms underlying these pathologic changes remain obscure. Our recent study characterized axon-targeted delivery of degradative lysosomes and demonstrated that the axon is an active compartment for local degradation (Farfel-Becker T et al., ***Cell Reports*** 2019). This work establishes a foundation for our current investigations into axonal lysosome trafficking and functionality in early NPC disease. Here we demonstrate that mature lysosome delivery to distal axons is significantly reduced in cortical neurons from Npc1 null mice, resulting in lower numbers of degradative lysosomes in NPC distal axons. Decreased axonal lysosome density leads to increased axonal autophagic stress that occurs without changes to autophagosome transport in NPC axons. The small GTPase Arl8b is

a limiting factor in coupling lysosomes to the kinesin-1 motor for driving lysosomal transport, and we demonstrate that elevated Arl8b expression facilitates axonal delivery of mature active lysosomes in NPC. Rescuing axonal lysosome density by Arl8b expression reduces autophagic stress in axons from pre-symptomatic NPC mice. Collectively, these observations suggest a new pathological mechanism by which impaired lysosome transport disrupts maturation and progression of the autophagy-lysosomal pathway and contributes to altered axonal homeostasis in NPC. (Supported by the Intramural Research Program of NINDS, NIH) Farfel-Becker T, Roney J C, Cheng X-T, Li S, Cuddy S R, and Sheng Z-H. (2019). Neuronal soma-derived degradative lysosomes are continuously delivered to distal axons to maintain local degradation capacity. ***Cell Reports*** 28, 51-64.

P1364/B495

Expression of Wipi2b Counteracts Age-related Decline in Autophagosome Biogenesis in Neurons.

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Autophagy defects are implicated in multiple late-onset neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS) and Alzheimer's, Huntington's, and Parkinson's diseases. Since aging is the most common shared risk factor in neurodegeneration, we assessed rates of autophagy in mammalian neurons during aging. We identified a significant decrease in the rate of constitutive autophagosome biogenesis during aging and observed pronounced morphological defects in autophagosomes in neurons from aged mice. While early stages of autophagosome formation were unaffected, we detected the frequent production of stalled LC3B-negative isolation membranes in neurons from aged mice. These stalled structures recruited the majority of the autophagy machinery, but failed to develop into LC3B-positive autophagosomes. Importantly, ectopically expressing WIPI2B effectively restored autophagosome biogenesis in aged neurons. This rescue is dependent on the phosphorylation state of WIPI2B at the isolation membrane, suggesting a novel therapeutic target in age-associated neurodegeneration.

P1365/B496

Defective Axonal Transport of Autophagosomes in *Drosophila* Models of Amyotrophic Lateral Sclerosis (als).

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder characterized by preferential death of motor neurons leading to muscle weakness and atrophy. Although the majority of ALS cases are sporadic, a G_4C_2 hexanucleotide repeat expansion (HRE) in *C9orf72* is the most common inherited cause of ALS (C9-ALS) and frontotemporal dementia (FTD). Since ALS displays selective vulnerability of motor neurons with cytoplasmic protein accumulation and a disruption of fast axonal transport, we hypothesize that dysregulation of axonal trafficking may be a critical step in pathogenesis of motor neuron degeneration in C9-ALS. Here, we performed in *vivo* imaging of *Drosophila* motor neurons expressing 30 G_4C_2 repeats (30R) and investigated the axonal transport of multiple organelles including mitochondria, dense core vesicles (DCVs), late endosomes (LEs), lysosomes and autophagosomes (APs). We found that 30R expression reduces the number of axonal APs, while it causes local accumulation of axonal DCVs, LEs and lysosomes with increased static population of organelles accompanied by alterations in retrograde transport. Since axonal transport of mitochondria was not affected by G_4C_2 HRE, we hypothesize that this preferential alteration in retrograde transport is

due to autolysosomal dysregulation rather than global disruption of axonal transport. Interestingly, 30R expression led to a marked reduction in AP number within motor neuron synaptic boutons, and this deficit was not restored by neuronal excitation with TrpA1. However, the remaining APs in C9-ALS motor neurons underwent normal maturation and development to amphisomes upon successful fusion with LEs during their axonal transport. Collectively, these results suggest that the G_4C_2 HRE causes an impairment in autophagosomal biogenesis in axons in C9-ALS/FTD, and further implicate protein quality control disruption related to autolysosomal degradation process as a critical step in pathogenesis of this disease.

P1366/B497

Stimulation of G_{aq}Promotes Stress Granule Formation.

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During adverse conditions, mammalian cells regulate protein production by sequestering the translation machinery in membraneless organelles (i.e. stress granules) whose formation is carefully regulated. Here, we show a direct connection between G protein signaling and stress granule formation through phospholipase Cβ1 (PLCβ1). In cells, PLCβ1, the most prominent isoform of PLCβ in neuronal cells, localizes to both the cytoplasm and plasma membrane. Here, we show that a major population of cytosolic PLCβ1 binds to stress granule proteins, such as PABPC1, eIF5A and Ago2. PLCβ1 is activated by $G_{\alpha q}$ in response to hormones and neurotransmitters and we find that activation of $G_{\alpha q}$ shifts the cytosolic population of PLCβ1 to the plasma membrane, releasing stress granule proteins. This release is accompanied by the formation of intracellular particles containing Ago2 aggregates, an increase in the size and number of particles containing PABPC1 and Ago2, and a shift of cytosolic RNAs to larger sizes consistent with cessation of transcription. These particles are seen when the cytosolic level of PLCβ1 is lowered by siRNA, osmotic stress or $G_{\alpha q}$ stimulation by carbachol. These stresses, in addition to cold, heat, oxidative and arsenite stress produces particles that appear to have different molecular compositions. Our results fit a simple thermodynamic model in which cytosolic PLCβ1 solubilizes stress granule proteins and its movement to $G_{\alpha q}$ upon stimulation releases these particles to allow the formation of stress granules. Taken together, our studies show a link between $G_{\alpha q}$-coupled signals and transcription through stress granule formation.

P1367/B498

Intracellular Trafficking and Secretory Defects in *Progranulin*-deficient Microglia.

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Microglia activation is a prominent feature in neurodegenerative diseases. However, there are many critical barriers in our knowledge regarding the cellular and molecular mechanisms that how the microglia are being activated and how these microglia interact with neurons to further promote neurodegeneration. Our previous work showed that deficiency in a common frontotemporal dementia (FTD) gene Progranulin (*GRN*) leads to age-dependent expansion in reactive microglia that promote excessive synaptic pruning in the thalamo-cortical circuit. To characterize the mechanism that promotes aberrant activation of microglia in the absence of progranulin deficiency, we performed stable isotope

labeling by amino acid in cell culture (SILAC) in *Grn*$^{+/+}$ and *Grn*$^{-/-}$ microglia and identified a number of proteins that were differentially expressed in *Grn*$^{-/-}$ microglia. An notations using KEGG pathways showed that most differentially expressed proteins in *Grn*$^{-/-}$ microglia were involved in the phagosome, AGE-RAGE signaling pathway, glutamatergic synapse, and cell adhesion molecules. Consistent with these results, *Grn*$^{-/-}$ microglia showed an increase in phagocytic activity of the zymosan particles and synaptic vesicles. In addition, *Grn*$^{-/-}$ microglia exhibited significant deficiency in cell adhesion properties to several extracellular matrix proteins, including collagens, laminin, fibronectin and fibrinogen. Since microglia is a secretory cell type, we asked how intracellular trafficking defects in *Grn*$^{-/-}$ microglia affected its secretory properties. To this end, we used ultracentrifugation to isolate extracellular vesicles from from *Grn*$^{+/+}$ and *Grn*$^{-/-}$ microglia. Nanosight analyses showed that loss of progranulin significantly reduced the secretion of exososmes in *Grn*$^{-/-}$ microglia. To further characterize how changes in secretion affected the cytotoxic properties in *Grn*$^{-/-}$ microglia, we collected serum-free conditioned media from *Grn*$^{+/+}$ and *Grn*$^{-/-}$ microglia and showed that *Grn*$^{-/-}$ microglia conditioned media were much more toxic to *Grn*$^{-/-}$ neurons. Using SILAC and quantitative proteomics, we showed that *Grn*$^{-/-}$ microglia conditioned media contained elevated amount of complements and lysosomal proteins. Together, these results provide critical insights into how progranulin deficiency alters a number of important cell biological properties in microglia, including endolysosomal trafficking, phagocytosis, cell adhesion and vesicle-mediated secretion. Changes in these properties most likely contribute to neurodegeneration in FTD caused by *GRN* mutations.

P1368/B499

Development of a *LYST*-deficient Glutamatergic Neuronal Model of Chediak-Higashi Syndrome.

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Chediak-Higashi Syndrome (CHS) is a lysosome-related organelle (LRO) disorder caused by bi-allelic mutations in the lysosomal trafficking regulator gene (*LYST*), which has 53 exons and encodes a 429 kDa protein (LYST). The clinical characteristics of CHS include oculocutaneous albinism, primary immunodeficiency, coagulation problems, risk for development of hemophagocytic lymphohistiocytosis and high risk of progressive neurological problems such as tremors, ataxia, weakness and intellectual disability. To date, several animal models of CHS have been investigated, but none of them consistently recapitulates the neurological phenotype seen in patients. Additionally, the role of LYST in lysosomal biology is poorly understood and likely a cell type-dependent. For these reasons, we aim to investigate the functions of LYST in a neuronal cell model. Here we describe the generation of a *LYST*-deficient neuronal cell model system using clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR interference technologies to respectively knock-out and knock-down the expression of endogenous *LYST* in induced pluripotent stem cells (iPSCs). These iPSCs were also engineered to facilitate an inducible expression of the transcription factor Neurogenin 2 to enforce their differentiation into glutamatergic neurons. With this approach, we have successfully established a *LYST*-deficient glutamatergic neuronal system to study the role of LYST in lysosomal biogenesis and trafficking through the endo-lysosomal pathway. Additionally, the homogenous population of neuronal cells obtained by this approach could potentially be utilized for global proteome, transcriptome and metabolome studies.

P1369/B500

Egfr Sorting Defects Cause Reduced Neural Stem Cell Proliferation and Microcephaly.

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The development of the neocortex is a highly regulated process whereby each cell type must be correctly specified and positioned. Alterations in this process can lead to a variety of cortical malformations, including microcephaly (small brain). This latter pathology has been recently related to mutations in the WD repeat domain 81 (*WDR81*) gene. WDR81 is a poorly characterized transmembrane protein that has been involved in endosomal maturation as well as aggrephagy. It remains unknown how mutations in WDR81 alters the growth of the neocortex. To address this question, we have generated a CRISPR/Cas9 WDR81 knock-out mouse. Mutant animals die perinatally and recapitulate the human microcephaly phenotype. We demonstrate that the reduced brain size is not due to increased apoptosis or altered cell fate, but to reduced neural stem cell proliferation rates. Importantly, patient-derived fibroblasts also display reduced proliferation, indicating that this may be a general feature of WDR81 loss of function. We identify alterations in the MAP kinase signalling pathway, which shows reduced ERK phosphorylation in mutant cells following EGF stimulation. We further demonstrate that this defect is due to a strong reduction of the EGR receptor (EGFR) expression levels. EGFR levels can be rescued following EGF starvation, indicating that this downregulation is due to altered EGFR downstream signalling. Indeed, we observe EGFR intracellular clearance delays following its internalization after EGF binding. We demonstrate that EGFR accumulates in aberrant swollen early endosomes in WDR81 patient mutant cells, which we also observe in mutant mouse neural stem cells in vivo. Together, this work identifies endosomal EGFR sorting defects as the cause of reduced neural stem cell proliferation and WDR81-linked microcephaly.

P1370/B501

The Ataxia-associated Gene Vps13d Is Required for Mitochondrial Fission and Mitophagic Progression in *Drosophila* Neurons.

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In order to support a healthy pool of mitochondria, neurons must sustain a balance of mitochondrial fission and fusion, along with the degradation of damaged mitochondria. Disrupting this balance, and consequently compromising mitochondrial function is a common theme in neurological and neurodegenerative diseases. Recent genetic studies in humans have associated mutations in *VPS13D* with ataxia and childhood-onset movement disorders, and uncovered an essential role for Vps13D in the regulation of mitochondrial morphology. While little is known about the function or cellular localization of Vps13D, its parental yeast protein (vps13) and metazoan family members (Vps13A and C) localize to inter-organelle contacts sites. Human mutations in all members of the Vps13 family (VPS13A-D) are associated with neurological disorders, sparking growing interest in understanding their function and mechanism. In order to better comprehend the vital role of Vps13 proteins in the nervous system, we examined the cell biological consequences associated with loss of Vps13D in *Drosophila melanogaster* neurons. We have found that targeted knockdown of Vps13D in larval motoneurons induces the formation of oversized mitochondria, and a subpopulation of these abnormal mitochondria are being

targeted for degradation through mitophagy. This subpopulation represents intermediates stuck in the late stages of the mitophagy pathway: post-polyubiquitination of damaged mitochondria, but prior to targeting to the lysosome. To understand this novel defect, we asked whether enlarged mitochondria generated via other mechanisms similarly initiate and stall in the mitophagy pathway. Knockdown of the mitochondrial fission protein Drp1 resulted in enlarged mitochondria; however, highly abundant mitophagy intermediates similar to Vps13D loss are only observed when Drp1 was removed in a null mutant background for core autophagy gene Atg5. These observations suggest that inhibition of fission alone induces active and complete mitophagy in neurons. In contrast, mitophagy intermediates in neurons lacking Vps13D stall at the stage of mito-phagophore elongation. Ultrastructural and IHC studies indicate that these intermediates rupture and lose matrix components to the cytoplasm. We conclude that Vps13D loss results in two progressive defects: first in mitochondrial fission, which induces mitophagy, and reveals a second defect in mitophagic progression. Both of these processes are critical to general mitochondrial health, likely contributing to the pathophysiology of the neurological diseases associated with mutations in Vps13D.

P1371/B502

***Hot* Gene Expression and Function in *Caenorhabditis ELegans*.**

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The Ly6 protein family is structurally characterized by the 4 highly conserved disulfide bonds, giving its members a “three-fingered” shape similar to that of alpha-neurotoxins found in snake venom. Despite these structural similarities, very little is known about the function of the Ly6 proteins. Studies of Ly6 function in mammalian systems are made difficult by the large size of the family, which contains 40 members. In *C. elegans*, however, there are only 10 members of this protein family, making it an apt model organism for establishing an understanding of the function of these proteins. As a first step to deducing the function Ly6 proteins, we hoped to learn their cellular location by generating transcriptional GFP reporters followed by fluorescence microscopy for each of the *hot* genes, as the genes that encode members of the protein family in *C. elegans* are called. In addition, we utilized RNAi knockdown in an attempt to study function. Thus far, we have successfully cloned GFP reporters for *hot* 4 and *hot* 7. Images of these strains show that these proteins are located near the pharynx, alluding to a potentially neuronal role for these proteins. Moving forward, we hope to determine the specific cell, or set of cells, where each hot gene is being expressed as well as gather more conclusive data regarding function using gene deletion analysis.

P1372/B503

Characterization of the HOPS/CORVET Complex in Axon Development.

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The transport of cargos along neuronal processes is important for maintaining neuronal health and function. This transport occurs in two directions; anterograde (towards axon terminals) and retrograde (towards the cell body). An terograde transport is accomplished by a super family of kinesin motor proteins while retrograde transport is accomplished by a single motor protein complex, Cytoplasmic dynein. While we know that dynein is responsible for all retrograde transport, how it attaches to specific cargos and moves them to the right place at the right time is largely unknown. This process is critical for neurons as disruption to retrograde transport is causal in many neurodegenerative diseases. To address

this gap in our knowledge, our lab uses forward genetics to identify novel modulators of dynein activity. In our forward genetic screen, we identified a mutant *vps18* zebrafish line. This mutant has axon terminal swellings, a sign of disrupted retrograde transport, and hypopigmentation along the body. Further tests show our *vps18* mutants have a loss of function mutation in the Vps18 protein. VPS18 (Vacular protein sorting-associated protein18) is a core component of the HOPS and CORVET complexes in yeast. The HOPS and CORVET complexes are important for membrane fusion in the endolysomal pathway. These two complexes share four proteins as part of the Class C Core subunits consisting of VPS18 along with VPS16, VPS33, VPS11. Each complex also has two complex specific proteins; VPS41 and VPS39 for HOPS, and VPS8 and Tgfbrap1 (VPS3) for CORVET. To determine if Vps18 defects were due to loss of the HOPS and/or CORVET complexes, we used a G0 crispant screen. Guide RNAs against individual HOPS components resulted in axon terminal swellings and hypopigmentation as seen in our *vps18* mutants. Conversely, knockout of CORVET-specific proteins Tgfbrap1 and Vps8 had no pigment or axonal phenotype. This indicates that HOPS, rather than CORVET, has a role in axonal physiology. Additionally, transport of various cargos such as autophagosomes, early and late endosomes and dynein was disrupted and their localization also altered compared to wild type fish, suggesting an additional role for the HOPS complex beyond vesicle fusion. As part of our future studies, we hope to discern the exact role of the HOPS with respect to transport of cargos in neurons and how this transport is important for maintaining neuronal health.

P1373/B504

3r:4r Tau Splicing Isoform Ratio Functions as a Leading Indicator for Tdp43 Proteinopathy.

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3R:4R Tau Isoform Ratio Functions as a Leading Indicator for TDP43 Proteinopathy Amyotrophic Lateral Sclerosis (ALS) is an upper and lower motor neuron disease characterized by progressive motor neuron degeneration. One common pathological hallmark in both familial and sporadic ALS is the presence of cytoplasmic inclusions of TAR DNA Binding Protein 43 (TDP43)—a highly conserved, predominantly nuclear protein that facilitates alternative splicing. Interestingly, these inclusions are also be found in frontotemporal dementia, Alzheimer's disease and chronic traumatic encephalopathy patient pathology. In order to better understand this pathology, the Donnelly lab has established an optogenetic system for rapidly inducing TDP43 inclusion formation with blue light that involves the coupling of a photo-active protein, Cryptochrome 2 (Cry2), to TDP43. The Cry-2 protein rapidly homo-oligomerizes when stimulated with blue light and quickly disaggregates when the stimulus is removed. This platform allows for spatiotemporal control by way of rapid inclusion formation and disaggregation at physiologically relevant translational levels. Furthermore, the platform induces the recruitment of endogenous TDP43 protein into the artificial inclusions thus sequestering TDP43 to the cytoplasm. Our lab has optimized this "OptoTDP43" construct in numerous cell lines and demonstrated that the induced inclusions recapitulate the biochemical markers characteristic of those observed in patient pathology. While our model permits the unparalleled control of insoluble inclusion formation, we have yet to characterize the etiology and pathway of cell death in our model. I hypothesize that the cell toxicity we observe in our model after chronic stimulation is not due to the insoluble cytoplasmic inclusions, but rather, the functional depletion of nuclear TDP43 and subsequent changes in the RNA splicing as a result. One target of TDP43 mediated splicing that is of interest is tau—a microtubule associated protein linked to numerous neurodegenerative pathologies. In recent years, researchers have identified that certain ratios of tau splice-isoforms are associated with a neurotoxic phenotype, particularly imbalances

in the ratio of 3R to 4R tau. It is the objective my research to translate the optoTDP43 model into a new neuronal cell line with GFP tagged TDP43 to demonstrate that cytoplasmic sequestration of TDP3 alters the 3R:4R tau isoform ratio to recapitulate an established tau pathological phenotype. Furthermore, using an oligonucleotide therapy designed to disaggregate cytoplasmic inclusions of TDP43, I wish to demonstrate that the neurotoxic tau isoform ratio can manifest independent of cytoplasmic TDP43 inclusion formation.

P1374/B505

Metabolic Regulation of Neuronal Mitochondria Via O-GlcNAcylation.

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The brain has an exceptionally high energy demand. Metabolic requirements vary greatly among brain regions, classes of neurons, individual neurons, and even neuronal sub-compartments. It is the job of mitochondria to provide 95% of the energy exactly when and where it is needed. The brain consumes glucose as the primary source of energy, therefore neurons are particularly sensitive to spatio-temporal glucose fluctuations. Our previous work showed that the metabolic sensor enzyme O-GlcNAc transferase (OGT) regulates mitochondrial positioning in neurons by sensing glucose availability. This OGT-mediated response is an adaptation that would allow mitochondria to concentrate in regions of high glucose. However, little is known about how O-GlcNAcylation affects neuronal metabolism and mitochondrial bioenergetics. Here, we demonstrate that high O-GlcNAcylation enhances mitochondrial bioenergetics by modifying key mitochondrial proteins in neurons. First, we observed changes in mitochondrial functions with maximal O-GlcNAcylation by inhibiting O-GlcNAcase (OGA) activity. Then, we utilized quantitative mass spectrometry to determine proteome that undergo O-GlcNAc regulation from isolated neuronal mitochondria. Total mitochondrial O-GlcNAcome and specific modification sites were mapped using higher energy collision dissociation (HCD) and electron transfer dissociation (ETD) fragmentation, respectively. Functional annotation on these proteins revealed key metabolic proteins including electron transport chain subunits, mitochondrial transmembrane transporters, and ketone body metabolizing proteins. These results suggest OGT alters mitochondrial function in adaptation to metabolic environment. We conclude that O-GlcNAcylation fine-tunes these subsets of proteins differentially, which eventually leads to higher glucose utilization. This study will lay a groundwork for how O-GlcNAcylation regulates metabolic homeostasis in neurons.

P1375/B506

Axonal Transport of an Insulin-like Peptide Mrna Promotes Stress Recovery In*C. Elegans*.

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Aberrations in insulin or insulin-like peptide (ILP) signaling in the brain causes many neurological diseases. Here we report that mRNAs of specific ILPs are surprisingly mobilized to the axons of C. elegans during stress. Transport of the ILP ins-6 mRNA to axons facilitates recovery from stress, whereas loss of axonal mRNA delays recovery. In addition, the axonal traffic of ins-6 mRNA is regulated by at least two opposing signals: one that depends on the insulin receptor DAF-2 and a kinesin-2 motor; and a second signal that is independent of DAF-2, but involves a kinesin-3 motor. While Golgi bodies that package nascent peptides, like ILPs, have not been previously found in C. elegans axons, we show that axons of stressed C. elegans have increased Golgi ready to package peptides for secretion. Thus, our

findings present a mechanism that facilitates an animal's rapid recovery from stress through axonal ILP mRNA mobilization.

Neuronal Degeneration and Regeneration 1

P1376/B507

Caspase-independent Structural Plasticity of the Actin-spectrin-based Membrane-associated Periodic Skeleton (MPS) Underlies the Initiation of Sensory Axon Degeneration.

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Axon degeneration shapes neuronal connectivity patterns during development and is an early hallmark of several adult-onset neurodegenerative disorders. Despite the substantial progress in identifying effector mechanisms driving axon fragmentation, much less is known about the upstream signaling pathways that initiate this process. Here, we investigate the caspase-independent structural plasticity of the actin-spectrin-based Membrane-associated Periodic Skeleton (MPS) in response to neurotrophin deprivation and the spectrin-dependent retrograde signaling in axon degeneration. We find that trophic deprivation (TD) of mouse sensory neurons causes a rapid disassembly of the axonal MPS, which occurs prior to protein loss and caspase activation. Besides, we show that actin destabilization initiates TD-related retrograde signaling needed for degeneration; actin stabilization prevents MPS disassembly and the retrograde signaling. Interestingly we also find that depletion of bII-spectrin, a key component of the MPS, suppresses retrograde signaling and strongly protects axons against degeneration. Our data demonstrate importance of structural plasticity of the MPS on initiation of axon degeneration. eLife, 8, p.e38730 (2019)

P1377/B508

Age-dependent Autophagy Induction After Injury Promotes Axon Regeneration Via Limiting Notch.

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Autophagy is essential for maintaining cellular homeostasis by the degradation of organelles or proteins. It also has a prominent role in modulating aging. But the role of Autophagy in the neuronal response to axon injury and subsequent axon regeneration, particularly in the context of aging, remains largely unknown. Our candidate genetic screen for axon regeneration regulators has identified genes in the autophagy pathway. Using a reporter that monitors autophagosomes and autolysosomes, we were able to monitor the dynamics of autophagy during axon regeneration. In response to axon injury, there was a significant increase in the number of autophagic vesicles. The injury-triggered autophagy activation and the capacity of axon regeneration undergo an age-dependent decline, and both of the declines were partially rescued by autophagy-activating agents. We found that DLK-1 was both required and sufficient for injury-induced autophagy activation. Autophagic vesicles were co-localized with Notch receptor LIN-12, a previously identified inhibitor of axon regeneration. Epistasis analyses indicate that LIN-12 might be a target of autophagy in axon regeneration. Taken together, our data suggest that DLK-mediated

injury signaling can activate autophagy, which might limit the level of LIN-12/Notch to promote axon regeneration. Our findings reveal that autophagy activation can promote axon regeneration in neurons that lack the maximal regrowth capacity, providing a promising therapeutic strategy for axon injury.

P1378/B509

Ubiquitin Proteasome Dysfunction as a Biomarker for Neurodegenerative Disease.

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The ubiquitin-proteasome system (UPS), chaperone mediated-autophagy (CMA), and mitophagy constitute the protein quality control system of the cell. Since damaged proteins are rarely repaired, the failure of protein quality control is particularly difficult to overcome in terminally differentiated cells (e.g., neurons). UPS dysfunction likely plays a key causative role in Alzheimer's disease (AD) and Parkinson's disease (PD); for example, mutations in the ubiquitin ligase parkin lead to loss of mitophagy, destruction of dopaminergic neurons and, thus, to Parkinson's Disease. Numerous developmental neuronal defects and neuromuscular pathologies are attributed to mutations in UPS genes. LifeSensors has studied the consequences of these mutations by screening brain tissues, serum, and CSF of healthy individuals (postmortem) and age-matched AD patients. Because antibodies against ubiquitin or target proteins are ineffective due to low affinity, epitope masking, and an inability to discriminate among various poly-ubiquitin chains, LifeSensors developed high-affinity chain selective polyubiquitin binding tools called TUBEs (Tandem Ubiquitin Binding Entities) to isolate ubiquitylated proteins. These enriched ubiquitylated protein fractions were analyzed by mass spectrometry-based proteomics to identify candidate markers for UPS dysfunction in neurons; comparing brain tissues from healthy individuals and AD patients, we uncovered numerous proteins with altered ubiquitylation patterns in the disease state. For example, the Tau protein is heavily ubiquitylated at multiple sites in AD, while Tau from normal brain is essentially ubiquitin-free; other unique ubiquitylated biomarkers were identified for AD. We are currently investigating CSF and plasma samples from AD and PD patients to establish a panel of unique ubiquitylated markers with reference to control samples. The goal of this study is to develop a diagnostic panel of ubiquitylation signatures using pan-selective and linkage-selective TUBEs for blood-based early diagnosis of AD/PD.

P1379/B510

Role of Aurora Kinase a and Endolysosomes in Alzheimer's Disease.

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Endolysosome dysfunction plays an important role in the pathogenesis of Alzheimer's disease (AD), the most common cause of dementia. The acidic pH of endolysosomes is not only critical for the activity of pH-sensitive enzymes involved in amyloidogenic processing of amyloid-β (Aβ) precursor protein but also is critical for endolysosome degradation of Aβ and aggregated Tau; even subtle de-acidification of endolysosomes accelerates amyloidogenesis and tauopathy. Because endolysosome acidification decreases amyloidogenesis and p-Tau, re-acidifying endolysosomes might represent a promising therapeutic strategy against AD. Recently, the activity of aurora kinase a (AURKA); a kinase that regulates vital neuronal functions including microtubule organization, synaptic plasticity, and neuronal migration was reported to be decreased in postmortem brain tissues of AD patients. However, virtually nothing is known about the expression levels and distribution patterns of AURKA in brain as well as the

role of AURKA in AD pathogenesis. Here, we tested the hypothesis that decreased AURKA activity results in endolysosome de-acidification and contributes to the pathogenesis of AD. Using immunofluorescence staining we showed that AURKA phosphorylation, which correlates positively with its activity, was significantly decreased in hippocampus of AD patients and in 3xTg-AD mice. Using immunoblotting and immunostaining methods, we demonstrated that AURKA was expressed in primary cultured rat neurons, in adult mouse brain, and in human brain tissues. Furthermore, using ratiometric measurements of endolysosome pH and ELISA measurements of Aβ, we demonstrated that activation of AURKA with anacardic acid resulted in endolysosome acidification and decreased levels of Aβ, whereas inhibiting AURKA with MLN8237 decreased AURKA phosphorylation, and increased levels of Aβ. Decreased AURKA activity may contribute to the pathogenesis of AD in part because of endolysosome de-acidification and re-acidifying endolysosomes using AURKA activators may provide for a new therapeutic strategy against AD. (Supported by MH100972, MH105329, MH119000 and DA032444)

P1380/B511

Late-breaking News: Autophagy Goes on Strike! - Rampant Immune Response Kills Neurons!.

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Autophagy and innate immunity are two homeostatic processes highly debated for their role in neurodegeneration (ND). How these two homeostatic processes plays role in age related neurodegenerative diseases are currently murky and in the focus of intense investigation. Here we examine the role of these two processes in cyclin dependent kinase (Cdk5) mediated neurodegeneration. Cdk5, an unusual cyclin-dependent-kinase, plays no role in cell cycle progression, but acts exclusively in postmitotic neurons, where its deregulated activity is linked to Alzheimer's and Parkinson's in humans. First, we observed hyperactivated innate immune response, particularly anti-microbial peptides (AMPs) in the brain of Cdk5-altered animal, using microarray profiling. We further examined the expression of AMPs in 3d-, 10d-, 30d- and 45d-old using qPCR and simultaneously counted dopaminergic (DA) neurons. We observe that Cdk5-associated upregulation of AMPs is accompanied by ND in age dependent manner. Conversely, targeted overexpression of individual AMP in DA neurons results in DA neuron loss in 30d-old normal flies. Finally, genetically reducing AMPs expression using a NFκB (Relish) mutant suppresses DA neuron loss in Cdk5-altered *Drosophila*. These data argue strongly that innate immune activation is necessary and sufficient for DA neuron loss in Cdk5-altered animals. Next, we asked why altered Cdk5 activity stimulates AMPs expression. The first clue in this regards come from our microaaray data, where we observed downregulation of components of autophagy, alongside the overactivation of innate immunity. Futher observation revealed high levels of Ref(2)P, the *Drosophila* p62, autophagosome accumulation, reduced level of cysteine protease-1 (Cp1) and vacuolar ATPase (Vha-13), confirming compromised autophagy in Cdk5-altered brain. Moreover, we found overexpression of AMPs in a mutant that blocks autophagy (*Atg8a*) strengthening the argument that reducing autophagy may overactivate innate immunity. Finally, we restored autophagy by moderate expression of the canonical autophagy regulator, TFEB (Mitf), in p35-null *Drosophila* brain and observed decreased AMP expression and rescue of DA neuron loss. These data suggest that it was reduced autophagy that stimulates AMPs overexpression in Cdk5-altered Drosophila. Our study recasts our picture or neurodegeneration by revealing a linear relationship between autophagy, innate immunity and neuron loss. Considering the conservation of genes and signaling pathways between flies and

mammals, these data open a new avenue for further exploration of the relationship between these two core processes in human neurodegenerative disease.

P1381/B512

The Role of Optineurin in Neuronal Mitophagy.

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Mitophagy, the selective removal of damaged mitochondria, is thought to be critical to maintain neuronal homeostasis. Mutations in proteins implicated in mitophagy, including PINK1, Parkin, OPTN, and TBK1, cause Parkinson's disease or ALS, suggesting defective mitochondrial turnover contributes to neurodegeneration. To test this hypothesis, we used mild oxidative stress to induce low levels of mitochondrial damage in hippocampal neurons. We observed the sequential recruitment of Parkin, TBK1, and OPTN to depolarized mitochondria followed by their sequestration into autophagosomes, and determined this pathway was compartmentally restricted to the soma. Further, acidification of mitophagosomes was remarkably slow in neurons and overall was a rate-limiting step in the mitophagy pathway. Expression of an ALS-linked OPTN mutation disrupted the integrity of the mitochondrial network and this effect was exacerbated by oxidative stress. We propose that the slow kinetics of mitophagy enhance neuronal susceptibility to disease-associated mutations in the pathway, leading to neurodegeneration.

P1382/B513

Neuronal Traffic Jams: Mechanistic Insights into Mutant Mammalian Prion Aggregate-mediated Intracellular Transport Impairments.

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A ubiquitous hallmark of neurodegeneration is the accumulation of misfolded protein aggregates inside axons. Intracellular aggregates can impair or block the proper transport of cellular components from the soma to the synapse, but the mechanisms of transport impairment remain unclear. Using an in *cellulo* neuronal system expressing a mutant prion protein (PrP^{PG14}) that forms aggregates inside axons and causes prion diseases in humans, we tested the hypothesis that PrP^{PG14} aggregates poison neurons by creating traffic jams to intracellular transport. Using high-resolution quantitative imaging approaches, we showed that PrP^{PG14} aggregates selectively interfere with the anterograde axonal transport of pre-synaptic vesicles and mitochondria by sequestering kinesin-1 away from cargoes, and thus disrupting the association between kinesin-1 and cargo adaptors as these complexes move through the aggregates. Our findings reveal a novel mechanism by which intracellular protein aggregates selectively disrupt axonal function and transport through sequestration of molecular motors. This study suggests potential targets for the development of strategies to combat intracellular prion aggregation, as well as the aggregation of misfolding-prone proteins involved in other neurodegenerative diseases.

P1383/B514

Evaluating Adc Related Peripheral Neuropathy with Human Ipsc-derived Neuronal Models.

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Antibody-drug conjugates (ADCs) are novel chemotherapeutics designed for more selective delivery of cytotoxic agents to cancer cells. However, toxicity of ADCs in normal cells has been reported in multiple

pre-clinical studies and clinical trials, leading to significantly narrowed safety margins. Peripheral neuropathy is a frequent adverse event with microtubule inhibitor (MTI) ADCs that can be challenging to assess in short term preclinical studies. For example, peripheral neuropathy was not predicted based on nonclinical toxicology studies in monkeys or rats treated with vcMMAE ADCs. MTI payloads MMAF and MMAE were tested alone and as ADCs linked to AB-095, an antibody against the non-mammalian tetanus toxoid protein, in two in *vitro* iPSC-derived neuronal models: 1) a 2D model from PhenoVista Biosciences in which test articles were administered to cells during neurite outgrowth and 2) a 3D model from Mimetas that examines test article effects on a pre-formed neurite network. Multi-parametric evaluation of imaging data resulted in ranking each test article's peripheral neuropathy potential as MMAE > AB095-MMAE > AB095-MMAF ≥ 2 (MMAF). This ranking is consistent with what can be expected based on clinical experience. Both 2D and 3D Human iPSC-derived neuronal models produced essentially the same test article ranking. Results indicated that the effect on neurite outgrowth formation and pre-formed neurite network integrity appear to provide the same outcome. Specifically, payload permeability appears to be important in ADC-mediated neurotoxicity in *vitro* and target-independent (non-specific) ADC uptake is unlikely to contribute significantly to neurodegeneration. Further testing of MTI ADCs/payloads in co-culture systems (sensory neurons and Schwan cells) and DRG sensory nerves (e.g.,Nerve on a Chip platform) may provide additional insight. All authors are employees of AbbVie and may own AbbVie stock. AbbVie sponsored and funded the study; contributed to the design; participated in the collection, analysis, and interpretation of data, and in writing, reviewing, and approval of the final publication.

P1384/B515

Parp1 Promotes Oligodendrocyte Remyelination in Cuprizone-induced Mouse Model of Multiple Sclerosis.

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Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) in which oligodendrocytes and myelin are primary targets. Promoting oligodendrocyte regeneration and myelin repair remains a major challenge in treating MS patients. Poly (ADP-ribose) polymerase 1 (PARP1) is a nuclear protein whose enzymatic activity plays an important role in DNA damage and repair. Previous studies reported that PARP1 and its activity are upregulated in demyelination lesion in MS brains. However, the role of PARP1 in myelin pathogenesis and repair remains elusive. We employ the cuprizone model to study the expression and function of PARP1 in oligodendrocyte regeneration and myelin repair. Our results showed that PARP1 and its enzymatic activity were increased in newly regenerated oligodendrocyte during myelin repair after cuprizone-induced oligodendrocyte damage and demyelination. Inhibiting PARP1's activity by small inhibitors decreased the rate of oligodendrocyte regeneration from oligodendrocyte progenitor cells and impaired timely myelin repair. Our study reveals that PARP1 activity is required for oligodendrocyte generation from oligodendrocyte progenitor cells and promotes remyelination in a toxin-induced demyelination animal model of MS. Our study also points to the potential of increasing PARP1's activity to promote oligodendrocytes regeneration and remyelination in treating MS patients.

P1385/B516

Knockdown of CTDSP1 as a Novel Strategy to Boost Neurotrophin Synthesis and Enhance Peripheral Nerve Regeneration After Traumatic Injury.

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Peripheral nerve injury (PNI) is a frequent complication of musculoskeletal trauma. Unfortunately, current treatments rarely restore complete motor and sensory function, with patients often experiencing long term deficits, such as motor dysfunction and chronic pain. Nerve regeneration and accurate reinnervation depend in part on the neurotrophic support received from surrounding cells at the zone of injury. We have previously identified a population of musculoskeletal trauma-induced mesenchymal progenitor cells (MPCs) that are found at high concentration at the site of injury, and exhibit neurotrophic properties. The expression of neurotrophins such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF) and neurotrophin-3 (NT-3), is repressed by a single transcriptional repressor known as RE1 silencing transcription factor (REST), which is protected from degradation by C-terminal domain small phosphatase 1 (CTDSP1). As direct targeting of transcription factors is challenging, we investigated whether modulating CTDSP1 activity is sufficient to promote REST degradation and stimulate the expression of neurotrophins. To characterize the expression profiles of REST, CTDSP1 and neurotrophins after musculoskeletal trauma, we quantified RNA and protein in traumatized muscle tissue and trauma-derived MPCs. qRT-PCR and Western Blot analysis showed that REST, CTDSP1 and neurotrophin expression is altered in a time-dependent manner following traumatic injury. Consistent with CTDSP1 role in stabilizing REST, an increase in CTDSP1 levels is associated with a decrease in neurotrophin levels. To investigate whether decreasing CTDSP1 activity increases neurotrophin production, we transfected cultured MPCs and dorsal root ganglion neurons with CTDSP1-targeting siRNA. qRT-PCR analysis of neurotrophin mRNA and ELISA quantification of secreted BDNF in cell culture supernatants showed that knockdown of CTDSP1 results in higher expression of neurotrophins and increased secretion of BDNF. Taken together, our results suggest that modulating CTDSP1 activity may represent a novel strategy to promote nerve regeneration and muscle reinnervation by increasing neurotrophin expression both proximally in the cell bodies of the injured nerves and distally in neuronal support cells at the site of injury.

P1386/B517

Major Histocompatibility Complex of Class I (MHC-I) Expression in Human Brain and Cultivated Astrocytes.

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Major histocompatibility complex of class I (MHC-I) plays a pivotal role in the adaptative immune response. It has also been implicated in neuroplasticity during development and after lesion. Indeed,

interferon beta-1b (IFN-beta) increases MHC-I expression by motoneurons after sciatic nerve crush in mice improving axonal growth and functional recovery. IFN-beta induces glial hypertrophy associated with upregulation of fibrillary acidic protein (GFAP) and MHC-I in astrocytes in *vitro*. Silencing of beta-2 microglobulin (β2M), a component of the MHC-I, decreases GFAP gene expression and downregulates astrogliosis in co-cultured astrocytes and spinal cord neurons, suggesting that MHC-I is associated with astrocyte activation. Even though MHC-I expression has been reported and functionally confirmed in murine nervous tissue, less is known regarding such expression and function in the adult human nervous tissue. Thus, we investigated MHC-I expression in the human brain, by using samples of non-epileptogenic anterior temporal lobe obtained as part of the surgical approach to treat hippocampal sclerosis (n=5). The cortical MHC-I expression was studied in histological sections and primary cultures of astrocytes derived from the same specimens. MHC-I in *vitro* expression was evaluated after exposing astrocytes to IFN-beta (100, 500 and 1000 IU/ml) for 5 days. MHC-I was mainly detected (β2M immunolabeling) in blood vessels, and in cortical neurons to a lesser extent. In *vitro* analysis showed expression of β2M in the control group (not exposed to IFN-beta), with consequent increase after IFN-beta stimulation. Flow cytometry quantitative data showed β2M upregulation ranging from 63% to 243%, in comparison to control. In *vitro* astrocyte activation was also investigated by the expression of vimentin, which presented up to 44% upregulation (100 IU/ml of IFN-beta), as compared to the control. Moreover, GFAP showed 34% upregulation (500 IU/ml of IFN-beta). In accordance with previous studies in rodents, our data show that IFN-beta is capable of increasing MHC-I expression, stimulating reactivity of human astrocytes. Considering that MHC-I and astrocytic activation may be protective after peripheral nerve damage and in neurodegenerative conditions, the current study opens perspectives for further research on the pathophysiological roles of MHC-I in the human nervous system in health and disease. Financial support: State of São Paulo Research Foundation (FAPESP) - 2017/03619-2; 2018/05006-0; 2013/07559-3.

P1387/B518

Response of SH-SY5Y Cells to N-(3-oxododecanoyl)-l-homoserine LactoneResponse of SH-SY5Y Cells to N-(3-oxododecanoyl)-l-homoserine Lactone.

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Inflammation is a pivotal mechanism in an organism’s response to physical or pathogenic stressors. The inflammatory process aids in the response of the immune system and the subsequent release of healing factors. Bacteria utilize hormone-like signaling molecules, known as quormones, to coordinate growth and increase their chances of successfully colonizing a host. There is growing evidence connecting these quormones to an inflammatory response. The purpose of our study was to determine if quormone n-(3-oxododecanoyl)-L-homoserine lactone (ODHL) could induce an inflammatory state capable of affecting the normal function of cellular model SH-SY5Y. After exposing retinoic acid-induced SH-SY5Y to low concentrations of ODHL, we found that their neurite connections weakened, their population numbers decreased, and inflammatory signals cyclooxygenase-2 and tumor necrosis factor alpha were expressed shortly after the exposure began. We believe that our data provides evidence to conclude that ODHL has the potential to cause a detrimental effect to nervous tissue in vivo.

P1388/B519

The Effects of Crispr Cas-9 System on Progeny of Hereditary Parkinson'S Disease in *C. Elegans*.

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We have studied the effects of gene editing on *Caenorhabditis elegans* expressing the mutation responsible for heritable Parkinson's disease. To do this, a strain of *C. elegans*, a nematode often used as a model organism, was acquired. This strain possessed a mutation in its lrk-1 gene which is a homologue for the LLRK-2 gene found in humans and is responsible for the development of hereditary Parkinson's disease. The clustered, regularly interspaced short palindromic repeats (CRISPR) Cas-9 system and RNA interface (RNAi) were applied to the Parkinson's disease strain of *C. elegans*. By doing this, the effects of the CRISPR Cas-9 system on the progeny of the nematodes were studied. The CRISPR Cas-9 system targets specific stretches along the genome and turns the desired gene on or off. This allows for permanent changes of the genes to occur. RNAi is a precise way to target specific genes in the genome. Once targeted, the RNAi is able to "fix" the genes of the individual. Combining the RNAi and CRISPR allows the "fixed" genes to pass down to the offspring of each generation. Some of the progeny of the Parkinson's strain of *C. elegans* did not express the lrk-1 gene, and therefore are free of hereditary Parkinson's disease. During the initial trial, 50 worms containing the Parkinson's disease gene were given the CRISPR and RNAi combination and they were scored for presence or absence of agglutination indicating a functional lrk-1 gene. In the long term, this method could be applied to humans that suffer from hereditary Parkinson's disease, and prevent the genes from being passed down to their successive generations.

P1389/B520

Neuronal Regeneration with Recelularized Nerve Allografts in Peripheral Nerve Injuries.

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INTRODUCTION. Peripheral nerve injuries (PNI) are one of the most frequent causes of motor and sensitive deficits in adults. The gold standard graft material is autograft. Nevertheless, certain disadvantages have been associated (limited length, donor site morbidity, and neuroma formation). Thus, alternatives such as decellularized and recelularized nerve allografts have been under development. The objective was to assess the regenerative capacity of a recelularized allograft (RA) with allogeneic mesenchymal stem cells derived from adipose tissue (AMSC-AT), pre-differentiated in vitro to Schwann-like cells (S-lC) in a PNI of an ovine model, compared to an autograft (AU). **MATERIALS and METHODS**. Ovine were randomly included in two groups of seven. At the operating room and under generalized anesthesia, a 3 cm resection in the left peroneal nerve (PN) was performed. In group AU, the PN was reversed, while in group RA, the allograft was previously treated within a fast decellularization method (FDM) based on sulfobetaines and chondroitinase and subsequently enriched with infiltrated S-lC. In both experimental groups, microsurgical neurorraphy technique was performed. Right PN was used as Sham group, the nerves were surgical identified and untreated. All surgical wounds were closed by planes. The nerve regeneration analysis was through histological stains: Hematoxylin and Eosin (H&E) for general evaluation, and Masson's Trichrome (MTC), to identify connective tissue nerve components. In addition, histochemistry with: Periodic Acid of Schiff (PAS), Klüver-Barrier (KB), and Marsland-Glees-Erikson (MGE) silver impregnation stains were performed; they selectively identify external lamina (EL),

myelin, and axons, respectively. All right PN were also procured as Control (C) group purpose. **RESULTS.** One year after surgery, morphological analysis revealed that AU group had better nerve fibers organization than RA group. Densitometric analysis on connective tissue revealed no statistically differences ($p>0.05$) between both experimental groups. Also, no differences were observed on EL in all groups. Furthermore, a significant ($p<0.05$) myelin increased densitomety in both experimental was identified, compared with C. Axons were significantly decreased in both experimental groups. **CONCLUSIONS.** Recelularized allografts proved to be a successful treatment for PNI, with comparable results as gold standard. Despite the less organization degree of nerve fibers, the recelularized allograft is capable of regenerate. Recelularized allografts are a therapeutic option in PNI since, unlike autograft, the need for a second surgery and nerve donor site morbidity are avoided. In addition, using S-lC in the acellular scaffold might decrease pre-surgical time in patients.

Establishing and Maintaining an Organelle 2

P1390/B522

Multiple C2 Domain Containing Proteins, Mctp1 and Mctp2, Regulate Lipid Droplet Biogenesis.

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Lipid droplets (LDs) are ubiquitous, dynamic organelles that serve as cellular storage sites for lipids. Nascent LDs form at specialized sites in the ER membrane, though little information is available about these subdomains. Previously, we showed that, in yeasts, both Pex30p and Pex31p have a reticulon-like transmembrane domain and Pex30p can tubulate liposomes in *vitro*. We found that Pex30p ER subdomains are sites of nascent LDs formation. In the absence of Pex30p, LDs are smaller and form clusters and exhibit decreased TAG levels. Also, deletion of *PEX30* and*SEI1* (which encodes the yeast homologue of the LD biogenesis factor Seipin) results in a severe growth defect, strongly suggesting that Pex30p plays an important role in LD biogenesis. Using a homology prediction program (HHpred), we found that the transmembrane region of mammalian proteins MCTP1 and MCTP2 exhibits homology to Pex30p. The MCTP proteins localizes to ER subdomains in a manner similar to Pex30p. Depletion of MCTP2 causes a decrease in the number and size of LDs in Hela cells and worms whereas overexpression leads to increase in the number of LDs. MCTP proteins are uniformly distributed in the ER when overexpressed. However, upon addition of oleic acid these proteins are re-distributed to ER-LD contact sites. We are currently determining the role of MCTP proteins in LD biogenesis.

P1391/B523

Investigating the Role of Lipid Droplet Sub-populations through Proteomic Landscape an alysis.

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Eukaryotic cells store fatty acids in the form of esterified lipids, triacylglycerol (TG) and sterol esters (SE), in cytoplasmic organelles called lipid droplets (LD). These organelles are of crucial importance to cellular metabolic homeostasis as they can serve as neutral lipid reservoirs for future energy utilization, and also sequester toxic fatty acids, preventing lipotoxicity. Previous mammalian and yeast studies have shown that LDs can exist in subpopulations with unique proteomes that heavily influence their function in maintaining cellular metabolic homeostasis (Schott et al., *JCB*, 2019; Eisenberg-Bord et al., *JCB*, 2018; Teixeira et al., *JCB*, 2018). Published work from our lab showed that, in yeast, a hallmark of nutrient scarcity is formation of a subpopulation of LDs at the Nucleus-Vacuole Junction (NVJ), a phenomenon

known as LD clustering (Hariri et al., *JCB*, 2018). Furthermore, we find that NVJ-associated LDs interact with the NVJ tether protein Mdm1, which we hypothesize helps to facilitate LD turnover. To investigate the function of this distinct LD subpopulation at the NVJ, I performed a proteomics screen of LDs constitutively clustered at the NVJ via Mdm1 overexpression. From this proteomic data, I selected several candidates that enrich on Mdm1-associated LDs at the NVJ. Currently, I am investigating the relationship between these candidate proteins and the extent to which they regulate LD homeostasis and spatial positioning, as well as how the contribute to metabolic homeostasis within the cell.

P1392/B524

Opa-1 Deficiency in Skeletal Muscle Increases Lipid Droplet Formation.

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Defective mitochondria-associated membranes (MAMs) or mitochondria and endoplasmic reticulum (ER) contact sites (MERCs), have been associated with Insulin-Resistant Type 2 Diabetes Mellitus (T2DM). MERCs are enriched with specific proteins and lipids that are believed to mediate inter-organellar communication such as calcium transfer and lipid transfer. T2DM subjects have miscommunication of lipid transfer inside of the cell and MAMs have been implicated in controlling these changes. MERCs dysfunction has also been implicated in fat accumulation in skeletal muscle, which has been shown to occur in insulin resistance. We have previously demonstrated that Optic Atrophy 1 (OPA-1) deficiency in skeletal muscle induces ER stress, which correlates with upregulation of Mitofusin-2 (MFN-2), a known tethering protein in MERCs. MFN-2 ablation has been shown to alter phospholipid metabolism and MERC tethering in murine skeletal muscle cells. Therefore, we hypothesized that OPA-1 deficiency increases the number of lipid droplets in skeletal muscle cells through an MFN-2 MERC related mechanism. To test this hypothesis, we surveyed for lipid droplets in primary OPA-1-deficient myotubes and lipid metabolites in OPA-1- deficient skeletal muscle. Primary myotubes were generated by isolating satellite cells from OPA-1 floxed mice, differentiating, and subsequently deleting OPA-1 by infecting the cells with adenoviral CRE recombinase. TEM image analysis of OPA-1-deficient myoblasts and myotubes confirmed an increase in the number of lipid droplets in cells. Conversely, ablation of MFN-2 in myotubes showed a decrease in the number of lipid droplets, as did the OPA-1/MFN-2 double-knockout. In addition, TEM image analysis of OPA-1 knockout cells showed an increase in smooth MERCs, and MFN-2 deletion increased rough MERCs distance in cells. Lastly, statistical analysis of lipid metabolites revealed that metabolite peak intensities were higher in OPA-1 knockout tissue than in wildtype tissue. Collectively, these data suggest that loss of OPA-1 in skeletal muscle cells results in an increase in lipid droplet formation and may result in an increase in lipid metabolite transfer in the MERC space. Notably, this increase may be due to the reduced smooth MERCs distance, as smooth ER has been shown to be more involved than rough ER in lipid transfer.

P1393/B525

Architecture of Interfaces between Lipid Droplets Revealed by Electron Cryo-tomography.

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Lipid droplets (LDs) are responsible for regulated storage and release of neutral lipids. LDs feature a neutral lipid core enclosed by a phospholipid monolayer and surface proteins involved in lipid metabolism. CIDE proteins govern the number and size of LDs in cells. CIDEC is crucial for fat storage in white adipocytes where it mediates fusion between LDs. This process is fundamentally different from fusion of bilayer-surrounded compartments. CIDEC accumulates at interfaces between LDs and facilitates directional neutral lipid transfer from the smaller to the larger LD. Deciphering the structure of the LD-LD interface is key in understanding the molecular mechanism of lipid transfer. Here, we use live cell imaging and electron cryo-tomography of vitrified human cells, thinned by focussed-ion beam milling. By live cell imaging we reveal that LD interface formation is a highly dynamic process. LDs contact each other repeatedly before eventually merging. Lipid transfer is completed within minutes. Our electron cryo-tomography shows that at the interface between LDs, the two monolayers are intact and in close apposition. LDs exhibit large-scale shape deformations and adjacent monolayers get as close as 5 nm. Depending on the size difference between the LDs, the interface morphology varies: i) minimal deformation, ii) flattening of LDs similar in size, or iii) smaller LD locally imposing curvature to larger LD. These differences possibly reflect distinct stages of interface formation. The interfaces contain a dense protein layer and apposing monolayers display waviness, indicating local packing disturbances. Currently, we use in *vitro* reconstitution to test if CIDEC is required and sufficient to establish the LD-LD interface architecture we observe in *situ*. Our results suggest that CIDEC-mediated neutral lipid transfer does not occur through a fusion pore formed between two LDs, but through close apposition of two intact monolayers. How the dynamics of contact formation exactly relate to the interface architecture remains to be seen. While the interface organization is conceptionally similar to known organelle contact sites involved in lipid transfer, the distance between the two monolayers is significantly shorter. Local disturbances in phospholipid packing possibly lower the energy barrier and allow neutral lipids to cross from one LD to the other.

P1394/B526

Drosophila Adipocytes Maintain Spatially Distinct Lipid Droplet Sub-populations.

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Adipocytes store nutrients as lipid droplets (LDs), but how they organize their LD stores to balance lipid uptake, storage, and mobilization remains poorly understood. Here, using *Drosophila* fat body (FB) adipocytes we characterize spatially distinct LD populations that are maintained by different lipid pools. We identify peripheral LDs (pLDs) that make close contact with the plasma membrane (PM) and are maintained by lipophorin-dependent lipid trafficking. pLDs are distinct from larger cytoplasmic medial LDs (mLDs), which are maintained by FASN1-dependent *de novo* lipogenesis. We find that sorting nexin CG1514/Snazarus (Snz) associates with pLDs and regulates LD homeostasis at ER-PM contact sites. Loss of *SNZ* perturbs pLD organization whereas Snz over-expression drives LD expansion, triacylglyceride production, starvation resistance, and lifespan extension through a DESAT1-dependent pathway. We

propose that *Drosophila* adipocytes maintain spatially distinct LD populations, and identify Snz as a regulator of LD organization and inter-organelle crosstalk.

P1395/B527

Cdc42 Negatively Regulates Endocytosis during Lumen Maintenance in Live Mice.

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Appropriate regulation of lumen establishment and maintenance are fundamental for tubular organs physiology. Cell culture model or small organism model have shown the detailed mechanisms regulating these processes, although little work has been done in mammalian model systems in *vivo*. In this study, we used the mouse salivary gland as a model system to investigate the role of the small GTPase Cdc42 to regulate the homeostasis of the intercellular canaliculi, which is a luminal structure of the acinar cells where saliva secretion occur. Cdc42 depletion in adult mice altered the morphology of canaliculi, whereas depletion at late embryonic stages inhibited the canaliculi formation. In addition, intravital subcellular microscopy revealed that Cdc42 depletion altered membrane trafficking from and towards the plasma membrane. In conclusions, we highlighted a novel role for Cdc42 to remodel the plasma membrane through the negative regulation of selected endocytic pathways.

P1396/B528

Endophilin A2-dependent Tubular Endocytosis Sustains Plasma Membrane Repair in the Absence of Caveolae and Promotes Cell Invasion by *Trypanosoma Cruzi*.

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Plasma membrane (PM) repair requires Ca^{2+}-triggered exocytosis of lysosomes, followed by lesion endocytosis[1]. Caveolae, PM invaginations that are abundant in injury-susceptible cells, participate in toxin pore (SLO) removal from the PM [2]. In B lymphocytes lacking detectable caveolin 1 (Cav1) or caveolae, SLO pore repair is mediated by Ca^{2+}-triggered lysosomal exocytosis and tubular endocytosis [3]. In this study we examined how Cav1-deficient cells process SLO wounds in the absence of caveolae, by comparing wild type (WT) and Cav1 knockout mouse embryonic fibroblasts (Cav1 KO MEFs). Cav1 KO MEFs were able to repair SLO wounds, but not as effectively as WT MEFs. Transmission electron microscopy (TEM) of cells treated or not with SLO in the presence of cholera toxin B (CTxB) showed an increase in CTxB-positive tubular endosomes in Cav1 KO MEFs. siRNA-mediated knockdown excluded the involvement of Galectin 3-dependent, clathrin independent carriers (CLICs) [4] in PM repair. The BAR-domain protein Endophilin A2 (but not A1 or A3), another regulator of tubular endocytosis [5], was essential for PM repair in Cav1 KO MEFs but not in WT MEFs. Knockdown of Endophilin A2 increased the number of open tubular endosomes observed by TEM, consistent with the proposed role for Endophilin A2 in membrane fission [6]. Since Endophilin A2 colocalized partially with endocytosed CTxB, we investigated its involvement in the PM deformation events that occur during cell entry by the parasite *Trypanosoma cruzi*, which subverts the PM repair machinery to invade host cells [7]. *T. cruzi* infection was partially inhibited in Cav1 KO MEFs when compared to WT, but Endophilin A2 silencing markedly decreased infection in both WT and Cav1 KO MEFs. Endophilin A2 was gradually recruited to nascent intracellular vacuoles containing *T. cruzi,* but was no longer associated with the parasites at later stages of infection. These results identify Endophilin A2 as a key component of the machinery for PM repair

that is subverted by *T. cruzi* for cell invasion ,and reveal a role for this BAR-domain protein in the tubular and clathrin-independent endocytic pathway that sustains PM repair in the absence of caveolae. 1 Idone, V. *et al. J Cell Biol* **180**, 905-914, doi:10.1083/jcb.200708010 (2008). 2 Corrotte, M. *et al. Elife* **2**, e00926, doi:10.7554/eLife.00926 (2013). 3 Miller, H. *et al. J Cell Biol* **211**, 1193-1205, doi:10.1083/jcb.201505030 (2015). 4 Lakshminarayan, R. et al. Nat. Cell Biol. 16: 595-606, doi: 10.1038/ncb2970 (2014). 5 Boucrot, E. *et al. Nature* **517**, 460-465, doi:10.1038/nature14067 (2015). 6 Renard, H. F. *et al. Nature* **517**, 493-496, doi:10.1038/nature14064 (2015). 7 Fernandes, M. C. *et al. J Exp Med* **208**, 909-921, doi:10.1084/jem.20102518 (2011). This work was supported by NIH R01 GM064625 to NWA.

P1397/B529

Membrane Dynamics and Regulators during Endosomal Tubulation.

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The endosomal system is a major sorting station consisting of various membrane compartments controlling many intracellular transport and signaling events. At early endosomes, the dynamic formation and release of narrow and highly curved tubular network, called recycling endosomes, enable the sorting and delivery of specific cargoes to target membranes. How the limiting membrane of endosomes is locally remodeled to ensure the generation of recycling tubules is still poorly understood. Here, we address how protein and lipids could coordinate the biogenesis of the recycling network by focusing on the 8-subunit protein complex BLOC-1 and endosomal phospholipids. By manipulating the phospholipids abundance or localization through various in vitro approaches from cryo-EM to live cell imaging and biochemical analyses in cells, we demonstrate that the formation, shape and release of recycling tubules rely on a BLOC-1/Phospholipid couple. We thereby elucidate crucial sequence of events during the biogenesis of recycling endosomes with potential consequences in some genetic disorders.

P1398/B530

Lipid Scramblase Tmem16k Is an Interorganelle Regulator of Endosomal Sorting.

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It is emerging that essential cellular processes can be coordinated via interorganelle communication at membrane contact sites (MCS) between distinct organelles. However, the identity and function of such membrane contact sites in maintaining cellular physiology remain an open scientific question. Here, we report that TMEM16K, an endoplasmic reticulum lipid scramblase linked to spinocerebellar ataxia (SCAR10), is a critical interorganelle regulator of the endolysosomal pathway. We identify endosomal transport as a major functional cluster of TMEM16K in proximity biotinylation proteomics analyses. TMEM16K forms contact sites with endosomes, interacting with the small GTPase RAB7 and phosphatidylinositol 3-phosphate (PtdIns(3)P). Our study further implicates TMEM16K lipid scrambling activity in endosomal sorting at these sites. Absence of TMEM16K leads to dysfunction of the endolysosomal pathway, which human disease point mutants cannot rescue, and progressive neuromuscular impairment in mouse models, consistent with observed human pathology. Thus,

TMEM16K-containing ER-endosome contact sites represent clinically relevant platforms for regulating endosomal sorting.

P1399/B531

The Pattern of Vacuole Inheritance and Biogenesis in *S. Cerevisiae*.

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In short, organelles are compartments found in all eukaryotic cells and carry out fundamental processes such as synthesizing, transporting, and degrading proteins, molecules, and lipids. In particular, the lysosome is responsible for storing and degrading proteins. However, in individuals affected by Tay-Sachs disease, the lysosomes found in the neurons are unable to degrade proteins properly. In turn, this leads the lysosomes to be filled with waste, swell and increase in size. This phenotype impairs the central and peripheral nervous system leading to symptoms such as neurodegeneration, loss of motor skills, and seizures. In addition, the ratio of the lysosome size to the cell size is perturbed. This suggests that maintaining proper organelle size is vital for the cell's overall health. A widely used model to study lysosomes are the yeast vacuoles. Previous research has shown that vacuoles maintain a vacuole size to cell size ratio as the cell increases in size, however this ratio is lost in individuals with Tay Sachs disease. In budding yeast, organelles are physically dragged from the mother cell to its daughter cell in a process called inheritance, thus it is necessary that the daughter cell receives the proper amount of organelles. Since the mother cell is losing its organelles to its daughter, it must make up for that loss through organelle biogenesis (the synthesis of new organelles). It is interesting to note that the vacuole size in the mother cell remains intact even as it is losing vacuoles to its daughter cell. This implies that vacuole inheritance and biogenesis are highly regulated processes, yet the regulation of vacuole size scaling remains poorly understood. I hypothesize that there is a pattern in which the cell regulates vacuole inheritance and vacuole biogenesis. Using a combination of fluorescence with 3-D microscopy and image analysis software, I will measure organelle (vacuole and peroxisome) inheritance and biogenesis in live yeast cells to determine the timing of the two processes. As hypothesized, current data is suggesting that vacuole inheritance occurs in a steady progressive pattern over time. Understanding vacuole inheritance may shed light onto elucidating the role organelles play in overall cell health, thus providing insight into lysosomal storage disorders such as Tay-Sachs disease.

P1400/B532

Intersectin-1 Interacts with the Golgin, GCC88, to Couple the Actin Network and Golgi Architecture.

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The Golgi apparatus is a dynamic organelle and, in vertebrate cells consists of individual Golgi stacks laterally fused together in a continuous ribbon-like structure. The modulation of the Golgi ribbon architecture has recently been shown to be associated with the regulation of cellular signaling pathways, and our ongoing studies are investigating how the architecture of the Golgi ribbon contributes to cellular processes. The conversion of individual Golgi stacks into a ribbon structure is a highly regulated process which relies on a balance between the actin and microtubule network. While the involvement of the microtubule network has been well defined, the role of the actin network is poorly characterized. Our previous studies have shown that a membrane tether/golgin known as GCC88, located at the trans-Golgi network (TGN), modulates the architecture of the Golgi ribbon in a dose-

dependent manner. Increased levels of GCC88 led to the loss of the Golgi ribbon whereas GCC88 depletion resulted in the formation of longer Golgi ribbons[1]. The aim of the current study was to identify the underlying mechanism for GCC88-mediated re-organization of the Golgi ribbon. We have shown that GCC88-mediated dispersal of the Golgi ribbon is an actin-dependent process and involves non-muscle myosin-IIA[2]. To further understand the role of GCC88 in this process, candidate interactors were identified through an in vivo enzymatic labelling approach known as BioID. We have identified the long isoform of Intersectin-1 (ITSN-1), which has GEF activity for the Rho GTPase Cdc42, as a novel interaction partner of GCC88; demonstrated its localization at the TGN; and its involvement in linking the TGN to the actin cytoskeleton. Golgi ribbon fragmentation has been reported in various neurodegenerative diseases and we have demonstrated the potential relevance of our findings in neuronal cells using a model of neurodegeneration. 1. Gosavi, P, Houghton, FJ, McMillan, PJ, Hanssen, E and Gleeson, PA (2018) J Cell Sci 131, jcs211987 2. Makhoul, C, Gosavi, P, Duffield, R, Williamson NA and Gleeson PA (2019) Mol Biol Cell. 30(3):370-386

P1401/B533

The Exocyst Complex Is a Component of Cytoplasmic Rods and Rings.

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Inosine monophosphate dehydrogenase (IMPDH) and cytidine triphosphate synthase (CTPS) are two rate-limiting enzymes involved in the catalysis of GTP and CTP, that in humans form into filamentous structures termed cytoplasmic rods and rings throughout the cytoplasm. Although cytoplasmic rods and rings have been described in multiple cell types, neither their formation nor their composition are well understood. Here we identify a novel localisation for the exocyst complex. Both antibody labelled and GFP-tagged EXOC8 localise to prominent rod and ring-shaped structures throughout the cytoplasm that are distinct from previously described localisations for the exocyst. Upon closer inspection, these structures resemble beads on a string, similar to the structure of IMPDH2-containing cytoplasmic rods and rings previously observed by cryo-EM. Mutations in the closely related enzyme IMPHD1 are a cause of inherited retinal degeneration, and mutation in EXOC8 is a candidate for pleiotropic syndromes where retinitis pigmentosa is part of the clinical presentation. Therefore, we tested whether EXOC8 and IMPDH2 have a physical association. GFP-Trap analysis identified the two proteins as putative novel binding partners, and this interaction was lost in a disease-linked EXOC8 variant. We propose that exocyst function at cytoplasmic rods and rings plays a role in regulating cyclic nucleotide metabolism, and that dysregulation may contribute to the molecular pathology of retinal degeneration.

P1402/B534

RASSF4 Functions at the Interface between Actin and Intracellular Vesicles to Regulate PI(4,5)P_2 Synthesis.

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The tumor suppressor RAS association domain family 4 (RASSF4) interacts with the small G protein ARF6 to regulate the level of phosphatidylinositol 4,5-bisphosphate (PI[4,5]P_2) important for a wide array of cellular functions. Nevertheless, the biological context of this RASSF4-ARF6-PI(4,5)P_2 pathway is not well understood. We found that actin dynamics controls RASSF4-ARF6 association at F-actin-associated intracellular vesicles, where RASSF4 activates ARF6 to stimulate PI(4,5)P_2 synthesis. Disruption of the

SARAH domain of RASSF4 resulted in an enhanced association with ARF6 at intracellular vesicles and an increased level PI(4,5)P_2 at the plasma membrane (PM). Additional data derived from acute manipulation and live-cell imaging experiments indicate that PI(4,5)P_2 produced at intracellular vesicles by the RASSF4-ARF6 pathway is delivered to the PM in an actin-dependent manner. Disruption of this intracellular PI(4,5)P_2 synthesis pathway in RASSF4 knockout cells resulted in reduced PM PI(4,5)P_2, enlarged cell size, disrupted actin cytoskeleton, and abnormal lysosomes. Consistently, knockdown of RASSF4 greatly suppressed neurite outgrowth in hippocampal neurons. Together, our study reveals a novel PI(4,5)P_2 synthesis pathway mediated by actin-regulated association of RASSF4 and ARF6 at intracellular vesicles. This RASSF4-mediated pathway is important for actin cytoskeletal integrity, lysosome homeostasis, and numerous PI(4,5)P_2-dependent physiological functions.

P1403/B535

A Conserved Lysosomal Switch Regulates Male Meiosis in *Drosophila melanogaster*.

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Gametogenesis involves a series of well-orchestrated events that ultimately produces viable reproductive cells. Recently, we found that lysosome activation is required for the meiotic maturation of oocytes (female gametes), suggesting that lysosome activity may be developmentally linked to meiotic progression during oogenesis. It is currently unclear whether lysosomes would be similarly regulated during the development of sperm (male gametes). Using the fruit fly *Drosophila melanogaster*, we have found that lysosomes are robustly activated in meiotic spermatocytes. Inhibition of lysosome activation results in the accumulation of early meiotic cells, which are often multinucleate and display abnormally large lysosomes. Interestingly, the multinucleate germ cells we observe upon loss of lysosome function resemble those seen in aged and diseased testes, highlighting lysosomes as likely points of control. We have evidence that a known lysosome regulator, Mitf/TFEB, is expressed in the testis only as spermatocytes enter meiosis. This observation hints at a potential signaling mechanism that could entrain lysosome activity to early sperm development. Collectively, these findings indicate that lysosomes play a critical role in spermatogenesis. They also support the conclusion that lysosome activation is tightly linked to meiotic progression in both male and female germ cells. Accordingly, these studies begin to describe a fundamental aspect of gametogenesis that is common to both sexes. Further analysis of lysosome activation during spermatogenesis will likely shed light on developmental events central to germline biology, while also providing relevant information on lysosome regulation and modes of action.

P1404/B536

Heterogeneity of Organelle Morphology in 2D Breast Cancer Cell Culture and 3D Bioprinted Breast Tumor Systems.

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Early endosomes function as a nexus between mitochondria and plasma membrane to regulate a wide variety of cancer-associated cellular processes, including receptor-mediated endosomal trafficking, signaling and iron homeostasis. Unravelling the complex relationships between these organelles and

breast cancer progression will provide new tools for cancer therapy and diagnosis. Since, 3D cell growth has been shown to affect organelle morphology, the analysis of the morphology and function of organelles in 3D tumor systems is the new frontier of cancer cell biology. Here, we tackled this challenge by studying early endosomes, a complex and dynamic organelle, and their interaction with mitochondria, in a comparative manner across a wide variety of 2D-culture cancer cells and 3D tumor systems. We combined laser-direct write (LDW) bioprinted 3D tumor systems with novel quantitative 3D imaging approaches to define and quantify the role of 3D tumor heterogeneity in endosome and mitochondria morphology as well as endosome-mitochondria contact regions. Several different breast cancer cell lines were grown in 2D cell culture, 3D spheroid systems using liquid overlay and size- and shape-controlled LDW bioprinted 3D tumor systems. Cells were subjected to immunostaining using anti-Tom20, a mitochondrial marker, and anti-EEA1, an endosomal marker, Airyscan high-resolution microscopy and image analysis using Imaris 3D visualization software coupled with statistical measures. Ten morphometric parameters were integrated to define the organelle diversity index (ODI), which is derived from clustering analysis based on Shannon diversity index for those different morphometric parameters. MDA-MB-231, an aggressive breast cancer cell line, showed high morphology heterogeneity of endosomes and their interaction with mitochondria when grown in 3D tumor systems in comparison to 2D cultured cells. 2D MDA-MB-231 cells displayed a similar endosomal and mitochondrial morphology only to some of the cells in the 3D systems. Thus, 2D MDA-MB-231 cells showed a more homogenous organelle morphology, whereas 3D MDA-MB-231 presented alternate organelle morphology, in which endosomes and mitochondria colocalized strongly in perinuclear circular rings. This morphology may indicate the initial steps of autophagy and/or mitochondria uncoupling. These results make evident the importance of determining subcellular organelle morphology in cancer cells grown in 3D culture systems. Altogether, our results demonstrate that LDW generates size- and shape-controlled 3D breast tumor systems that can be used to specifically evaluate the role of 3D growth in the morphology of early endosomes and their interaction with mitochondria.

P1405/B537

Identifying Novel Proteins That Mediate Very-Low-Density Lipoprotein (vldl) Biogenesis and Trafficking in Liver Cells.

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Lipoproteins deliver lipids to organs and tissues through the blood stream. They play critical roles in lipid metabolism and homeostasis. The regulation of the lipoprotein biogenesis is critical because uncontrolled production is associated with diseases such as atherosclerosis and obesity. Lipoproteins are primarily generated in the liver, which produces VLDL, and the intestine, which generates chylomicrons. VLDL and chylomicrons are assembled in the ER lumen and mature as they traffic through the cell. They are structurally similar to lipid droplets, containing a core of neutral lipids surrounded by phospholipids and proteins. The major protein on VLDL and chylomicrons is apolipoprotein B (apoB), which is thought to be co-translationally lipidated, forming pre-VLDL particles that mature into VLDL particles before secretion out of cells. Lipidation requires microsomal triglyceride transfer protein (MTP) but is not well understood and probably requires other unknown proteins. To follow VLDL formation and trafficking in hepatocytes and to undertake a screen for proteins required for VLDL biogenesis, I have tagged endogenous apoB with GFP using CRISPR/Cas9. Because adding the gene encoding GFP can be challenging for the relatively large size of apoB, I used the recently devised strategy of tagging proteins

with a fragment of GFP that can form fluorescent GFP when it encounters the rest of the protein. I tagged apoB at the N-terminus with the 11th β strand of GFP (GFP11). The remainder of GFP, β strands 1 to 10 (GFP1-10), was expressed in the ER lumen by a lentivirus. When GFP11-apoB encounters GFP1-10 in the ER lumen, a fluorescent GFP-tagged apoB is formed. I have confirmed that apoB was successfully tagged with GFP using this scheme and the cells with the GFP-apoB were sorted and amplified. An additional advantage of this approach is that only ApoB outside the cytoplasm is visualized, allowing us to better track ApoB trafficking. We are going to use this cell line for CRISPR screening and diverse biochemical assays to find novel factors affecting the VLDL biogenesis and trafficking in the liver cells. We also successfully GFP-tagged apoE, another protein on VLDL, using the same method. These cell lines will allow us to study the lipoprotein biogenesis and screen for new proteins required for formation and trafficking of VLDL. Long-term, my hope is that these studies will open new targets for therapies or medicines to control lipoprotein production to lower the risk of the cardiovascular disease, which is the leading cause of death globally.

P1406/B538

Structural Basis of Curvature Generation by Dynamin-related Protein 1.

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Fission dynamin proteins are large GTPases that polymerize to remodel membranes. Their association with membranes and their propensity to polymerize have challenged efforts to understand the mechanism of their action. Specifically, the mechano-chemical details of how nucleotide binding, hydrolysis, and release are coupled to dynamin polymerization and membrane fission remain poorly understood. Using a disease allele of a fission dynamin family member, DRP1, which polymerizes to constrict and divide mitochondria, we stabilized a potential reaction intermediate of the DRP1 fission process. Here we report a 3D cryo-EM structure of a 12-membered ring of DRP1 with dimensions consistent with inner mitochondrial membrane fission and outer mitochondrial membrane “super-constriction”. Notably, we observe a previously undescribed interface between the nucleotide-bound GTPase domain and bundle-signalling element of one ring protomer and the stalk domain of the next protomer. This interface is blocked by a DRP1 receptor, MiD49, in a previously published structure of a linear DRP1-MiD49 copolymer. Compared to the linear copolymer, we document a sliding motion between protomers about interface 3, causing significant rearrangement of interface 1. Together, these changes describe how DRP1 can transition from a metastable, linear oligomer to a preferred, high-curvature oligomer in the absence of GTPase domain dimerization.

P1407/B539

Three-dimensional Live Imaging of Keratinocytes during Cornification Using Epidermal Equivalent Model.

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The stratum corneum plays an important role for water-impermeable barrier function. The process of cornification which granular cells transit to corneocytes requires for formation of the functional stratum corneum. Since corneocytes are quite different from granular cells, changes on the cells during cornification would be dynamic. However, the spatiotemporal details of these changes were still unclear. Here, we performed three-dimensional live imaging during cornification in an epidermal

equivalent model using a two-photon microscope with various fluorescent probes labeling intracellular structures. Our observation revealed that the granular cells during cornification not just simply flattened but temporally expanded before flattening. The morphological change from the expansion to the complete flattening took about 20 minutes. We also found that acidic vesicles which had been moving before cornification abruptly stopped just before the morphological change started. Focusing on mitochondrial morphology and amount, the granular cells with lower quantity of mitochondria, which were mostly punctate, tended to transit to corneocytes. Time-lapse imaging during cornification showed that remaining fragmented mitochondria were depolarized at the same time as the acidic vesicles stopped. After flattening, DNA signals were leaked from the nuclear region and gradually faded out. Furthermore, we also experimented using an epidermal equivalent model with impairment of filaggrin expression. The results of imaging suggested that filaggrin affected on a certain step of the morphological change. Our study would contribute to elucidate the mechanism of the cornification and skin diseases such as vulgar ichthyosis or atopic dermatitis.

P1408/B540

Optimizing Cell Factories: Uncovering the Role of Vacuolar Size in Biochemicals Yield.

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The organelles of eukaryotic cells house molecules involved in specialized biochemical reactions and thus, may be used as bioreactors. In this study, we focus on the vacuoles of *Saccharomyces cerevisiae* since they are dynamic organelles that serve crucial functions including pH and ion homeostasis, protein and organelle degradation, and storage. In addition, vacuoles have been suggested to hold the potential to manufacture compounds that can be converted to liquid fuels such as gasoline. However, it remains unknown if the size of an organelle can determine the quantity of biochemicals stored within them in addition to how vacuolar size could impact the quantity of compounds vacuoles can store. Therefore, the aim of this study is to examine if vacuolar size influences the quantity of compounds stored, which can provide insights on its capacity to yield compounds. Our compound of interest is 5-Aminoimidazole ribonucleotide (AIR), an intermediate molecule in the '*de novo*' Purine Biosynthesis (DNPB) pathway. AIR accumulates in the vacuoles of *S. cerevisiae* mutants defective in the *ADE2* gene (also involved in the DNPB pathway) when grown in adenine-deprived medium. Interestingly, AIR emits red fluorescence naturally when exposed to oxygen, thus, allowing us to visualize the content of the vacuoles and draw a relationship against vacuolar volume using fluorescent microscopy. Therefore, to study the capacity of different vacuole sizes to store AIR, we imaged *ade2* mutants via confocal, fluorescent microscopy. These images were used to reveal three-dimensional information of the vacuoles and AIR content from fluorescent signatures. The fluorescence intensities from the vacuolar membrane (GFP-tagged) and AIR were analyzed with two softwares, ImageJ and MatLab, and used to determine whether a correlation exists between vacuole size and AIR accumulation. Preliminary results suggest a positive correlation between vacuolar size and the amount of AIR accumulated. Understanding how vacuolar size affects the amount of a compound accumulated within the vacuoles could shed light on finding effective ways to enhance yeast vacuoles as bioreactors to manufacture other compounds of interest in larger scales.

P1409/B541

Proposed Plasma Membrane Sphingolipid Sensor Nce102 Regulates Ergosterol-dependent Processes of the Vacuolar Membrane in Yeast.

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Biomembranes are segmented into lateral microdomains of specific composition and function. This enables spatio-temporal segregation of not only diverse cellular processes, but also distinct biological functions executed by individual membranes. One of the best characterised microdomains of the yeast plasma membrane is the ergosterol-rich MCC (membrane compartment of Can1). It is stabilized by the eisosome, a cytoplasmic protein scaffold, which gives the microdomain its typical furrow-like shape. Chief among the core MCC constituents is the tetraspan protein Nce102. Its absence causes a decrease in number of MCC microdomains in the plasma membrane and renders them flat. While the exact function of Nce102 remains unknown, the protein has been proposed to act as a sensor of plasma membrane level of sphingolipids, and to be involved in regulation of their biosynthesis. We demonstrate that as the yeast culture ages, and the sphingolipid content of cellular membranes increases, a subpopulation of Nce102 migrates from the plasma membrane to the vacuole. In contrast to other plasma membrane proteins, this transition does not trigger degradation of Nce102. Instead, the protein is stably localized to ergosterol-rich domains of the vacuolar membrane, including the nucleus-vacuole junction, suggesting functional relevance. Indeed, deletion of *NCE102* and/or its functional homologue *FHN1* leads to several vacuolar phenotypes, prime examples being delayed vacuolar fusion (a hallmark of both cell ageing and filamentous growth) and less efficient formation of ergosterol-rich domains in the vacuolar membrane. Our data further suggest that the deletion strains might have a defect in lipophagy, a process directly dependent on pre-existence of ergosterol-rich domains in the vacuolar membrane, and vital for ergosterol recycling. Interestingly, both vacuolar fusion and lipophagy involve close apposition of membranes enriched either in ergosterol or sterol esters. The nucleus-vacuole junction is also an ergosterol-rich membrane contact site, as is the inside of the MCC furrow. All of these membrane domains are strongly favoured by Nce102. We therefore speculate that Nce102 might play a role in stabilization of membrane contact sites of ergosterol-rich membranes, which would explain why there are fewer eisosomes and flat MCC microdomains in *nce102*Δ strains. In this respect, Nce102 may function analogous to claudins and occludins, which are major stabilizers of tight junctions in animal cells. Consistently, Nce102 is structurally similar to claudins and occludins in that they all have four membrane spanning domains. Studies on Nce102 are hence expected to bring new insights into the roles of tetraspan proteins in the organization of eukaryotic membranes.

Peroxisomes and Chloroplasts

P1410/B542

Peroxisomes Contain Internal Membrane Compartments in Arabidopsis.

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Peroxisomes, which house vital and often oxidative metabolic reactions in eukaryotes, are typically described as small spherical organelles with a single delimiting membrane. Although occasional peroxisomal membrane complexity has been observed in yeast, mammals, and plants, technical challenges have limited the recognition and understanding of this complexity. We developed fluorescent

reporters to simultaneously label Arabidopsis peroxisomal membranes and lumen, and we examined these lines using live-cell imaging. Exploiting the unusually large size of Arabidopsis seedling peroxisomes, we discovered extensive and pervasive peroxisomal inner membranes. We found that peroxisomal internal vesicles accumulate over time, can harbor distinct proteins, use ESCRT (endosomal sorting complexes required for transport) machinery for formation, and are likely derived from the outer peroxisomal membrane. This unanticipated structural complexity necessitates revisiting the classical view of peroxisomes as simple, single-membrane bound organelles.

P1411/B543

Misdirection of Miro to Peroxisomes Reveals That the GTP-bound State of the N-terminal Domain Is Required for Motor/ Adaptor Complex Assembly.

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Mitochondria are actively transported in all somatic cells, including neurons and their proper distribution is essential for cellular function. Disrupted mitochondrial trafficking is linked to axon degeneration and dysfunctional synapses. Mitochondrial transport relies on microtubules and a highly regulated motor/adaptor complex that consists of four critical components: Miro, a mitochondrial outer membrane protein; Milton/TRAK1/2, the adaptor protein; and kinesin-1, and dynein, the molecular motors. Miro comprises a C-terminal transmembrane anchor in the outer mitochondrial membrane and two GTPase domains (N-GTPase and C-GTPase) that are separated by EF hands. Although the GTPase domains of Miro are a prominent feature, their functional significance is controversial. Overexpressing GTP and GDP-locked versions of Miro have been confounded by the presence of endogenous Miro and detrimental consequences of gross reorganization of the mitochondria. To circumvent these confounding factors, we have misdirected Miro1 to peroxisomes with the transmembrane domain of PEX3 and have expressed this construct in both COS7 cells and hippocampal neurons. This construct recruits to peroxisomes both coexpressed TRAK and kinesin-1. Using the peroxisome-directed Miro1, we find that the Miro1 N- GTPase regulates the ability of Miro1 to interact with the rest of the motor adaptor complex: neither TRAK1/2 nor kinesin colocalize on peroxisomes with the GDP-locked T18N mutation and the motility and distribution of the peroxisomes in COS7 cells and hippocampal neurons are correspondingly affected. The complex assembles correctly with the GTP-locked N-GTPase (P13V). In contrast, neither the GTP-locked nor GDP-locked C-GTPase mutations substantially altered Miro1 recruitment of TRAK1/2 or kinesin to peroxisomes. In hippocampal neurons peroxisomes bearing the P13V Miro1 or wildtype Miro1 are transported out of the soma into axons and dendrites, but those bearing T18N Miro1 remain in the soma. Thus, N-GTPase of Miro1 is critical for regulating Miro1's interaction with the other components of the motor/adaptor complex and thereby for regulating mitochondrial motility.

P1412/B544

Characterization of a Novel Peroxin in *Trypanosoma Brucei*.

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Peroxisomes are ubiquitous membrane bound organelles with multiple functions including the degradation of hydrogen peroxide and the oxidation of fatty acids. Kinetoplastids, a group of flagellated

protozoans, have specialized peroxisomes called glycosomes that are essential. Glycosomes are unique in that they compartmentalize the first seven steps of glycolysis and are indispensable. Despite their importance, we know little about the processes that regulate these organelles. Proteins known as peroxins (Pexs) coordinate peroxisome and glycosome biogenesis. While their function is often conserved across species, their sequences usually are not. Pexs regulate many processes including peroxisome/glycosome formation and proliferation, and the import of membrane and matrix proteins. In kinetoplastids, homologs for only a small number of known Pexs have been characterized, suggesting that there are more Pexs to be discovered. We are interested in identifying and characterizing additional Pexs. Pex19 is a soluble chaperone that delivers peroxisome membrane proteins, many of which are Pexs, to the peroxisome via interactions with the glycosome membrane protein Pex3. We queried the trypanosome genome for open reading frames that contain a Pex19 binding domain that is present yeast, mammal, and plant in Pex3. We found a single ORF (Tb927.9.11350) containing a putative Pex19 binding domain (P19BD; SNKLEIWEDLKIISFTR), which is conserved in all kinetoplastids. In pursuit of resolving the function of this putative Pex19 binding protein, Pex19BP, we have defined its cellular localization, purified organelles using epitope-tagged Pex19BP, and identified putative Pex19BP binding proteins via co-immunoprecipitation (co-IP). In the kinetoplastid parasite, *Trypanosoma brucei*, immunofluorescence assays revealed that staining for hemagglutinin antigen-tagged TbP19BP (HA-TbP19BP) overlapped with the glycosome protein aldolase. Western analysis of organelles isolated via immunoaffinity using HA-TbP19BP revealed the presence of multiple glycosome proteins and co-IP experiments demonstrated that TbP19BP interacts with proteins involved in glycosome protein import. Phenotypic characterization of Pex19BP-deficient cells is ongoing and will provide insight into the role this protein plays in glycosome biogenesis. Inhibition of glycosome protein import is lethal to trypanosomes. The identification of additional Pexs in these parasites provides a foothold into future investigations into the pathways that coordinate glycosome biogenesis, which may be exploited for pragmatic gain.

P1413/B545

Functional Peroxisomes Are Necessary to Maintain Regulatory Pathways of Cholesterol Homeostasis and Efficient Cholesterol Synthesis in CHO Cells.

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Cholesterol biosynthesis is a multi-step process involving more than 20 enzymes in several subcellular compartments. The pre-squalene segment of the cholesterol/isoprenoid biosynthetic pathway is localized in peroxisomes. Cellular cholesterol homeostasis involves the sensing of sterol levels and an appropriate response by altering the balance between dietary uptake, endogenous *de novo* synthesis, efflux, and (in liver) conversion to bile acids. Cells adjust their sterol content by both transcriptional and post-transcriptional feedback regulation of sterol metabolism, and the sterol regulatory element-binding protein (SREBP) family of transcription factors is central for this regulatory system. Using the peroxisome-deficient *Pex2* knockout mouse we have defined the importance of peroxisomes in maintaining normal cholesterol homeostasis. We showed that peroxisome deficiency activates ER stress

pathways in the liver, especially the integrated stress response, leading to dysregulation of the endogenous sterol response mechanism and SREBP-2 pathway induction. In this study, we compared the transcriptional regulation of cholesterol biosynthesis in wild-type Chinese hamster ovary (CHO-K1) cells and in three isogenic peroxisome-deficient CHO cell lines harboring *Pex2* gene mutations. Peroxisome deficiency activated expression of cholesterogenic genes; however, cholesterol levels were unchanged compared to wild-type CHO-K1 cells. HMG-CoA reductase (HMGCR) activity was significantly decreased in the mutant cells, whereas the protein levels of HMGCR, mevalonate kinase, and IPP isomerase (IDI1) were significantly increased. The rate of cholesterol synthesis was significantly reduced in peroxisome-deficient cells. U18666A, which inhibits cholesterol export from lysosomes, induced the mRNA and protein levels of cholesterol biosynthetic enzymes in peroxisome-deficient cells; however, cholesterol synthesis rates were significantly lower than in CHO-K1 cells. ER stress, mTORC1 and MAPK pathways were not involved in the SREBP-2 pathway activation in peroxisome-deficient CHO cells. Interestingly, peroxisome deficiency promoted SCAP trafficking from the ER to the Golgi even when cells were cultured in medium containing 10% FCS. Restoration of functional peroxisomes in peroxisome-deficient CHO mutants upon complementation with *Pex2* cDNA normalized the transcriptional regulation of the cholesterol biosynthetic pathway, the rate of cholesterol synthesis, and SCAP trafficking. These results highlight the importance of functional peroxisomes for maintaining regulatory pathways of cholesterol homeostasis and efficient cholesterol synthesis in CHO cells.

P1414/B546

Characterization of the Kinesin KSE2 in Stromule Formation and Function.

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Chloroplasts play an important role during plant immunity and are the source for many cellular signals during the defense response. During the immune response chloroplasts send out stroma-filled tubular extensions called stromules. Stromules are induced during both PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). Stromules extend along microtubules, and since kinesins are the only MT motors in plants, we hypothesized that a kinesin provides the motive force for stromule extension and stromule-driven movement. Our lab took a candidate approach to identify the kinesin for stromule extension by overexpressing kinesins belonging to kinesin family 14. One of the candidates resulted in increased stromule length and faster chloroplast movement, which we named kinesin for stromule extension 2 (KSE2). We are currently characterizing KSE2 to understand its role in stromule formation and function. To do this, we overexpressed 9 deletion mutants in *N. benthamiana in* which we deleted various domains of KSE2. These deletions include the actin-binding calponin domain (ΔCH), the coiled-coiled 1 domain (ΔCC1), both the ΔCH and ΔCC1 (ΔCH-CC1), only the motor (motor), no motor (Δmotor), the coiled-coiled 2 domain (ΔCC2), the C-terminal tail (Δtail), both the CC2 and tail (ΔCC2-Tail). The full length and ΔCC1 showed significant changes in stromule branching. The Δmotor showed no significant differences in stromules per chloroplast and branches per stromule. The full length KSE2, only the motor, ΔCC1 showed significant differences in stromules per chloroplast. All 6 deletion mutants tested showed significant differences in average stromule length. We are currently in the process of examining changes in stromule morphology for ΔCH, ΔCC2, and ΔCC2-Tail, and analyzing movement dynamics, such as extension velocity, for all KSE2 overexpression deletion mutants. This data suggests that KSE2 may be the motor required for stromule extension and future experiments will focus on examining knockdown and knockout mutants during plant innate immunity.

P1415/B547

The Study of Stromule Dynamics during Reactive Oxygen Species Regulation of Intra- and Inter-cellular Defense Signaling.

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Stromules, are stroma-filled tubular extensions of the chloroplast that appear to play a major role in cellular signaling. Specifically, they appear to impact the movement and localization of chloroplast as part of the regulation of immune response signals such a hydrogen peroxide (H_2O_2). However, the exact mechanics behind their function and precise role in signal transmission remains unclear. Chloroplast and stromule movement dynamics are dependent on the plant cytoskeletal networks. Stromule tips move along microtubules during extension, while chloroplast moment itself is dependent on the actin microfilament network. By utilizing viral induced gene silencing (VIGS) in *Nicotiana benthamiana,* it is possible *to* disrupt chloroplast movement by silencing a gene called Chloroplast Unusual Positioning 1 (*CHUP1*), which has been observed playing a major role in blue light mediated avoidance. An alysis of *CHUP1*-silenced *N. benthamiana* indicate that CHUP1 plays a regulatory role in the movement of chloroplast bodies, influencing the rate of chloroplast movement, as well as influencing the movement dynamics of stromules. While stromules have a vital role in intracellular communication, they may also function to send signals intercellularly via plasmodesmata (PD). The regulation of PD permeability limits the region of cell death as part of the hypersensitive response programed cell death (HR-PCD). In order to understand the role of signal transmission during HR-PCD it is necessary to understand the contribution chloroplasts and stromules play in the transmission of H_2O_2 as it relates to (PD) permeability. Preliminary data shows that during HR-PCD induction, there is an increased association of PD with both stromules and chloroplast bodies. This suggests that chloroplast generated signals are necessary for the regulation of PD permeability during HR-PCD. Determining the means of stromule and chloroplast movement regulation and their role in signal transmission will help elucidate the mechanics of the plant innate immune response. By understanding this, our ability to combat pathogens will only increase, as we will better be able to target the pathways involved in plant defense and disease resistance.

P1416/B548

Non-canonical Interactions in Intracellular Transport: Investigating the Physical Mechanisms of Hitchhiking and Tethering.

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Intracellular transport plays an important role in metabolism, signaling, cell growth, and development. The traditional picture of transport in cells involves the bidirectional movement of cargo attaching directly to cytoskeletal motor proteins such as kinesin and dynein, which move along microtubule tracks. In contrast to this canonical model, recent evidence shows that cargos such as peroxisomes, lipid droplets, mRNPs, and the endoplasmic reticulum can also move by attaching to other motor-driven organelles, navigating the cytoplasm through “hitchhiking”. A molecular component of hitchhiking has

been identified for peroxisome transport in *A. nidulans* fungal hyphae, where PxdA proteins on the surface of early endosomes are required for hitchhiking. However, the physical principles underlying this transport mechanism remain unexplored. We describe a quantitative model of hitchhiking, focusing on the interplay between the carrier organelle, linker protein and the transported cargo to obtain the rate at which hitchhiking can be initiated. Our work shows that transport initiation rates depend on the distribution of the cytoskeletal tracks and the carrier organelle, as well as linker protein stiffness. In mammalian cells, there is evidence that peroxisomes are tethered to microtubule tracks when not being actively transported. We have imaged peroxisomes in the hyphae of live *A. nidulans* cells and analyzed their motion to identify signatures of tethering to microtubules. Using an analytical model of fluctuating semiflexible polymers, we compared tethered peroxisome motion in live cells to the mechanical properties of the intracellular environment. Our computational model of hitchhiking predicts that tethering to microtubules results in a ten-fold enhancement of the hitchhiking initiation rate in geometries similar to fungal hyphae. This enhancement is expected to substantially increase the overall transport rate of hitchhiking organelles, lead to a more uniform distribution, and reduce the time required for hitchhiking cargo to encounter different cellular regions. Our results leverage a quantitative physical model to highlight the importance of organelle interactions in non-canonical intracellular transport and provide new experimental directions that will allow us to further elucidate this under-appreciated transport mechanism.

P1417/B549

Targeting of the Membrane-anchored Rab GAP (GTPase Accelerating Protein) Gyp8 to Peroxisomes Is Regulated by the AAA ATPase Msp1.

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Eukaryotic cells use vesicular transport to build and maintain membrane-bound organelles housing specific biochemical functions. Rab GTPase signaling proteins (Rabs) are key regulators of vesicular transport, controlling where and when membranes dock and fuse, ensuring lipid and protein cargoes are delivered to appropriate destinations. Rabs adopt their signaling active conformation when bound to GTP, allowing Rab interactions with tethering and membrane fusion proteins. Rabs depend upon GTPase accelerating proteins (GAPs) to trigger GTP hydrolysis and return the Rab to its GDP-bound, inactive state. Rab GAPs contribute to the efficiency and fidelity of transport pathways, and defective Rab GAPs are implicated in a variety of human diseases. Rab GAPs tend to be cytosolic proteins that transiently localize at membranes to survey for their client Rab. We report that the yeast Rab GAP Gyp8 is an atypical transmembrane (TM) GAP whose association with peroxisomes is regulated by the AAA ATPase Msp1, a chaperone that functions to remove tail-anchored proteins from peroxisomes and mitochondria. Computational analysis of Gyp8 predicted a single-pass TM domain near the carboxy terminus, characteristic of a tail-anchored protein. Fluoresence microscopy indicated that GFP-tagged Gyp8 co-localized with endoplasmic reticulum (ER) and peroxisomal markers in wild type cells. In the absence of peroxisomes, GFP-Gyp8 redistributed to the ER. Loss of Msp1 chaperone function resulted in mislocalization of GFP-Gyp8 to mitochondrial membranes. Subcellular fractionation biochemically demonstrated that Gyp8 localized exclusively to a membrane fraction containing peroxisomes. Truncation analysis of Gyp8 indicated that the TM and luminal domains are necessary and sufficient to direct localization to peroxisomes. These data represent the first report in any experimental system of a

Rab GAP that localizes to peroxisomes. Ongoing studies continue to explore the physiological significance of Gyp8 in regulating peroxisome dynamics.

P1418/B550

Ypk9, a Homolog of Parkinson'S Associated Park9, Has a Role in Peroxisomal Proliferation.

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Yeast PARK9, a homolog of human ATP13A2, was previously identified as a suppressor of alpha synuclein toxicity. Mutations in ATP13A2, a putative divalent cation transporter located in lysosomes, have been implicated in the aging disorder, Parkinson's disease. Because oxidative damage accumulates during aging, we speculated that loss of YPK9 may compromise growth and oxidants, such as hydrogen peroxide, may exacerbate it. We found the growth rates of *ypk9* and a wildtype strain to be nearly identical. However, growth of *ypk9* was severely impaired in the presence of hydrogen peroxide. When treated with hydrogen peroxide, the number of peroxisomes remained unchanged in wildtype cells but increased as much as 6-fold in YPK9 deletion strains suggesting that YPK9 plays a role in peroxisomal proliferation. DNM1, FIS1, and VPS1 were previously shown to regulate peroxisomal proliferation (fission). To test the hypothesis that YPK9 genetically interacts with DNM1, FIS1, and VPS1, we created a series of single and double deletions and monitored growth and the number of peroxisomes. The growth of *dnm1*, *fis1*, and *vps1* strains was modesty slower than the wildtype. Growing the strains in the presence of hydrogen peroxide further hampered growth. The *dnm1 ypk9* strain grew slightly faster and the *fis1 ypk9* strain grew slightly slower than the wildtype. The growth of *vps1 ypk9* strain was dramatically impaired. The *dnm1* and *fis1* strains had about the same number of peroxisomes as the wildtype, whereas the *vps1* strain had 25% fewer peroxisomes. Hydrogen peroxide increased the number of peroxisomes in *fis1*, *dnm1*, and *vps1* relative to untreated cultures. Although the number of peroxisomes in the *vps1 ypk9* strain was ~60% greater than the wildtype, the number in *dnm1 ypk9* and *fis1 ypk9* strains was about the same as the wildtype. Treating *fis1 ypk9* and *vps1 ypk9* with hydrogen peroxide increased the number of peroxisomes by more than 30%, whereas the number of peroxisomes in the *dnm1 ypk9* strain remained essentially unchanged suggesting that peroxisomal proliferation resulting from the loss of YPK9 is dependent on DNM1. Together, these results suggest that YPK9 negatively regulates peroxisomal proliferation and plays an important role in hydrogen peroxide detoxification.

P1419/B551

Investigating the Role of Peroxisomes in Hutchinson-Gilford Progeria Syndrome.

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Peroxisomes are small, membrane-enclosed organelles that house various enzymes with metabolic functions. One important feature in both Hutchinson-Gilford Progeria Syndrome (HGPS) and normal aging is the elevated levels of Reactive Oxygen Species (ROS), which are generated from metabolic pathways with the capacity to cause oxidative damage to macromolecules within the cells. Although peroxisomal bioreactions can generate free radicals as their byproducts, many metabolic enzymes within the peroxisomes play critical roles as ROS scavengers, in particular catalase. Preliminary data from our group showed multiple peroxisomal defects in HGPS fibroblasts. First, we observed impaired peroxisomes-targeting proteins trafficking, which suggested that the poorly assembled peroxisomes

might cause high oxidative stress, contributing to the premature senescent phenotype in HGPS. We then investigated the ROS clearance efficiency by peroxisomal enzymes, and found significantly decreased expression of catalase in HGPS. Furthermore, we evaluated the effects of two promising HGPS-treatment drugs Methylene Blue and Rad001 on peroxisomes in HGPS fibroblasts. We found that comparing to Rad001, Methylene Blue reduces cellular ROS with less peroxisomal toxicity. Our findings will bring new insights on the cellular aspects of HGPS as well as normal aging.

P1420/B552

Structural Investigation of *Arabidopsis Thaliana* Peroxins: Purifying PEX4 and PEX22 for Crystallization.

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Peroxisomes are essential organelles that host metabolic reactions such as the breakdown of fatty acids. Peroxisome function and biogenesis are controlled by proteins called peroxins (PEX). Structural data exist for several peroxins from organisms such as *Saccharomyces cerevisiae* and *Homo sapiens*, but no structures for *Arabidopsis thaliana* peroxins have been solved. PEX4 is an E2 ubiquitin conjugating enzyme that ubiquitinates peroxisomal membrane proteins. PEX22 anchors PEX4 to peroxisomes with a transmembrane domain. Interestingly, PEX22 is necessary for the function of PEX4 beyond its role as a membrane anchor, but this relationship is incompletely understood. *Arabidopsis* PEX4 has high sequence similarity to homologs in other organisms, but *Arabidopsis* PEX22 has virtually no sequence similarity to PEX22 proteins in other organisms. We are investigating the structure of *Arabidopsis* PEX4 in complex with PEX22. We have generated a construct of PEX4 linked to a soluble portion of PEX22 with a protease cleavage site. We have expressed these constructs in *E. coli* with a 6xHis tag and an MBP tag and have purified the proteins for crystallization. This research will further our understanding of peroxin structure in *Arabidopsis* and elucidate the role of PEX22 in PEX4 function.

4

Mitochondrial Quality Control

P1421/B553

The Lon Protease Promotes Mitochondrial Homeostasis in Lysosome Impaired Cells.

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Communication between organelles is crucial for cells to sense, respond, and adapt to environmental changes. Amongst organelle interactions, the mitochondria-lysosome connection is uniquely important due to the role of these organelles in regulating energy metabolism, nutrient sensing, and protein and metabolite recycling. Disruption of mitochondria-lysosome communication contributes to numerous diseases, including heart failure, neurodegeneration, diabetes, and inborn errors of metabolism. Working in budding yeast, we recently found that lysosomes promote mitochondrial respiration by spatially compartmentalizing amino acids. This sequestration is necessary to prevent amino acids from interfering with iron homeostasis, which is critically important for maintaining mitochondrial respiration. Here, we sought to identify pathways that operate to maintain mitochondrial homeostasis during amino acid-induced iron limitation. To do this, we performed genetic screens in yeast to identify genes that become conditionally essential in the absence of a functioning lysosome. These screens uncovered numerous genes important for maintaining mitochondrial homeostasis during lysosome impairment, including several mitochondrial localized proteases. Amongst mitochondrial proteases, we found that

the LON protease, a mitochondrial matrix-localized protease conserved from bacteria to mammals, becomes critically important during times of lysosome failure. Indeed, we found that both well-characterized and previously unknown LON substrates are degraded upon lysosome impairment, and demonstrate that degradation of these substrates during lysosome dysfunction is triggered by amino acid-induced iron limitation. We are currently working to determine how LON-dependent degradation of mitochondrial matrix proteins promotes mitochondrial health. Interestingly, many LON targets under these conditions are iron containing proteins. Based on this observation and the fact that their degradation is triggered by amino-acid induced iron limitation, our current model is that LON is required to prevent deleterious accumulation of apo, iron-less containing misfolded protein during times of lysosome impairment.

P1422/B554

Molecular Mechanism of Ubiquitin-dependent Mitophagy.

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Parkin and PINK1, two gene products mutated in a familial Parkinson's disease, have been identified as essential proteins for ubiquitin-dependent degradation of damaged mitochondria through autophagy machinery called mitophagy. Parkin is a member of the RBR E3 ligase harboring a RING1 domain for interaction with E2 enzymes and a RING2 domain for conjugation to ubiquitin molecule. However among 40 different E2 enzymes encoded in the human genome, E2 enzymes, which are recruited to the damaged mitochondria with Parkin during mitophagy, remain largely unknown. Here we developed a method in which interactions between E2 and activated Parkin on the damaged mitochondria are captured in cells, and identified many E2 enzymes required for Parkin-dependent mitochondrial ubiquitylation.

P1423/B555

Bridging the Gap between Energy Sensing and Mitophagy: Characterization of the Regulation of Mitophagy Receptors by Ulk1 and Tbk1.

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AMPK is a highly-conserved energy sensor, the master regulator of cellular metabolism, and has been shown to regulate mitochondrial autophagy via activation of its substrate, ULK1. However, the precise mechanism of AMPK-regulated mitophagy remains to be elucidated. The LC3-Interacting Region (LIR) is a conserved amino acid sequence present in some proteins that allows their binding to Atg8 family proteins, critical proteins for autophagosome formation. A subgroup of LIR-containing proteins called mitophagy receptors function to tether damaged mitochondria to the Atg8-decorted autophagosome. Previous studies have shown that phosphorylation at the serine before the conserved LIR sequence in some LIR-domain containing proteins increases the affinity of the LIR motif to LC3B and alters the function of the LIR-containing protein. For example, ULK1 has been reported to phosphorylate the mitophagy receptor FUNDC1 at one such serine, increasing FUNDC1 and LC3B binding affinity and promoting mitophagy. Interestingly, TBK1 has been reported to phosphorylate other LIR-domain containing proteins. Here we have investigated more broadly whether individual LIR-domain containing autophagy/mitophagy receptors are regulated by ULK1 or TBK1, or both, and the role that AMPK-dependent energy sensing plays in the regulation.

P1424/B556

Trpm2/ca^{2+}/camkii Pathway Leads to Impairment of Mitophagy to Mediate Neuron Cell Death in Models of Stroke.

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Ischemia neuronal injury has been demonstrated to induce mitochondrial dysfunction and mitochondrial reactive oxygen species (ROS) production, and mitophagy in turn is activated to repair dysfunctional mitochondrial and relieve mitochondrial ROS. Yet, whether and how ROS-sensitive TRPM2 channel (transient receptor potential cation channel subfamily M membrane 2) regulates mitophagy remains unknown. Here, we mechanistically studied the role of TRPM2 in oxygen and glucose deprivation-reperfusion induced mitophagy regulation. We find that TRPM2-mediated Ca^{2+} influx leads to mitophagy inhibition, which leads to death of primary neuron. Genetically knockout of TRPM2 channel or Chemically chelation of Ca^{2+} induces mitophagy and reduces cell death. Moreover, in response to OGD-Rep, TRPM2-mediated Ca^{2+} influx induces phosphorylation of CAMK2, and the activated CAMK2 subsequently reduces the translocation of parkin to mitochondria. Chemically inhibition of CAMK2 increases mitophagy and improves mitochondrial functions. Therefore, our results demonstrate that ROS activates the TRPM2/ Ca^{2+}/CAMKII/ cascade to decrease parkin translocation to mitochondrial leading to mitophagy impairment in models of stroke.

P1425/B557

A Close-up View of Mitophagy Using Mt-Keima and Fluorescence Lifetime Microscopy.

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Mitophagy is a cellular process that selectively removes damaged, old or dysfunctional mitochondria. Defective mitophagy is thought to contribute to normal aging and to various neurodegenerative and cardiovascular diseases. Previous methods used to detect mitophagy in vivo were cumbersome, insensitive and difficult to quantify. We created a transgenic mouse model that expresses the pH-dependent fluorescent protein mt-Keima in order to more readily assess mitophagy. Keima is a pH-sensitive, dual-excitation ratiometric fluorescent protein that also exhibits resistance to lysosomal proteases. At the physiological pH of the mitochondria (pH 8.0), the shorter-wavelength excitation predominates. Within the acidic lysosome (pH 4.5) after mitophagy, mt-Keima undergoes a gradual shift to longer-wavelength excitation. In addition to intensity imaging we describe here how to apply mt-Keima fluorescence lifetime microscopy (FLIM) to visualize mitophagy in live cells as well as various tissues including skeletal muscle, heart, liver, and kidney, obtained from mt-Keima transgenic mice. We observed that in control live cells mt-Keima fluorescence exhibits two components a short (0.4ns) lifetime corresponding to the mitophagic compartment and a longer (2.6ns) lifetime corresponding to normal mitochondria, in good correspondence to the intensity images. Interestingly, in the tissues the lifetime measurements reveal a heterogeneous mitophagic compartment containing in addition to the short (0.5ns) lifetime mt-Keima species an intermediary (1.2ns) longer lifetime component. Whether these 2 components correspond to different folding states, digestion products of the mt-Keima in the acidic environment remains to be elucidated. In conclusion FLIM provide a complementary approach to

asses mitophagy in normal cells and tissues as well as in disease situations, or altered under environmental, genetic perturbations, or in aging.

P1426/B558

Neuronal Mul1-Mfn2 Nexus Protects Stressed Mitochondria from Parkin-mediated Mitophagy through ER-Mitochondria Contacts.

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Chronic mitochondrial stress associates with major neurodegenerative diseases. As neurons are post-mitotic cells, recovering stressed mitochondria, rather than acute degradation via mitophagy, constitutes a critical step for mitochondrial quality control and thus energy maintenance in early stages of neurodegeneration. Our previous studies in mature cortical neurons revealed that mitophagy is observed in a small proportion of neurons following mitochondrial depolarization and that Parkin translocation to depolarized mitochondria occurs much more slowly than in non-neuronal cells (Cai et al., ***Current Biology*** 2012; Lin et al., ***Neuron*** 2017). These findings argue for intrinsic neuronal mechanisms that can maintain mitochondrial integrity before Parkin-mediated mitophagy is activated. To support this assumption, we address two fundamental questions: (1) Do distinct mechanisms are in place in neurons to act as a checkpoint for recovery versus rapid degradation of chronically stressed mitochondria? (2) Is mitophagy the second resort for neuronal mitochondrial quality control after recovery mechanisms have failed? by addressing these questions, we reveal a new mechanism through Mul1-Mfn2 pathway that acts as a checkpoint to maintain neuronal mitochondrial health under mild stress conditions (Puri et al., ***Nature Communications*** in press). Deficiency in mitochondrial Mul1 E3 ubiquitin ligase increases Mfn2 activity that triggers the first phasic mitochondrial hyperfusion and also acts as an ER-mitochondria (ER-Mito) tethering antagonist. Reduced ER-Mito coupling leads to increased cytoplasmic Ca^{2+} load that activates calcineurin and induces the second phasic Drp1-dependent mitochondrial fragmentation and mitophagy. Overexpressing Mfn2, but not Mfn1, mimics Mul1-deficient phenotypes, while expressing PTPIP51, an ER-Mito anchoring protein, suppresses Parkin-mediated mitophagy. Consistently, expressing Drp1 mutant or blocking calcineurin activity locks mitochondria in hyperfusion status in Mul1-deficient neurons. Thus, by regulating mitochondrial morphology and ER-Mito contacts, Mul1-Mfn2 pathway plays an early checkpoint role in maintaining mitochondrial integrity. Our study provides new mechanistic insights into neuronal mitochondrial maintenance under stress conditions, which is relevant to major neurodegenerative diseases associated with chronic mitochondrial dysfunction and altered ER-Mito interplay *(Supported by the Intramural Research Program of NINDS, NIH). Puri R, Cheng X-T, Lin M-Y, Huang N, and Sheng Z-H.* ***Nature Communications*** *(2019) (in press). Lin M-Y, Cheng X-T, Tammineni P, Xie Y, Zhou B, Cai Q, and Sheng Z-H.* ***Neuron*** *(2017); 94, 595-610. Cai, Q., Zakaria HM, Simone A, and Sheng Z.-H.* ***Current Biology*** *(2012); 22, 545-552.*

P1427/B559

Dysfunction of Hepatic Mitochondria in Alpha-1 Antitrypsin Deficiency Mediated Liver Disease.

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Alpha-1-antitrypsin deficiency (AATD) is an inherited disease characterized by emphysema and liver disease. AATD is most often caused by a single amino acid substitution at amino acid 342 in the mature protein, results in Z mutation of the AAT gene (ZAAT). This substitution is associated with misfolding and the accumulation of ZAAT in the Endoplasmic reticulum of hepatocytes and monocytes, causing a toxic gain of function. Mitochondrial injury in AATD mediated liver disease has been shown by altered morphology of mitochondria as well as mitochondrial autophagy in the liver of AATD individuals and in a transgenic mouse model ofAATD. In this the main objective of this study is to determine the role of mitochondrial dysfunction in the pathogenesis of AATD-mediated liver disease. Pi*M (normal variant) and Pi*Z (mutant variant) human AAT transgenic mice and PiMM and PiZZ hepatocyte cell lines were used to isolate mitochondria and cytoplasm fractions. The presence of AAT within the mitochondria was determined by western blot analysis, electron and immunofluorescence microscopy.The expression of genes and proteins related to glucose and lipid metabolism pathways were investigated by qPCR, western blot and immunofluorescent microscopy. Live cell metabolic assays were performed using a Seahorse XF analyzer.Pi*Z transgenic mice and PiZZ hepatocyte contain misfolded ZAAT in the hepatic mitochondria as well glucose and lipid metabolism dysregulation.We demonstrate that misfoldedZAAT is imported into mitochondria in our model systems. As a consequence of this transport the is an overabundance of misfolded proteins inside mitochondria that disrupts mitochondrial biosynthetic activities leading to organelle dysfunction. This novel mechanism has the potential to lead to novel interventions to prevent liver disease in AATD.

P1428/B560

A Mitochondrial Presequence That Drives Sorting to the Cell Surface in a Regulated Manner.

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Most of mitochondrial proteins are synthesized in the cytosol as precursors bearing an N-terminal presequence that acts as targeting signal. The hydroxyacyl-CoA deshydrogenase trifunctional multienzyme complex α-subunit (HADHA), involved in β-oxidation, has also been found at the cell surface, known there as gastrin binding protein (GBP). GBP binds gastrin with low affinity and mediates autocrine mitogenic effects in normal gastric and colon carcinoma cells. The mechanism addressing HADHA/GBP from the cytosol to either the mitochondria or the cell surface remains unknown. Interestingly, cytosolic HADHA has recently been described to interact with LC3, a protein crucial in the macroautophagic processes that remove cytosolic components and are increased during starvation conditions. Here, we study whether the presequence of HADHA has sorting information to target the protein to the cell surface and whether this process is regulated by starvation. We raised an antibody against a synthetic peptide of GBP detected variable levels of this protein at the surface of different tumoral cell lines by biotinylation assays. HADHA/GBP is an integral membrane protein as revealed by its resistance to sodium carbonate extraction. This antibody also showed the expected mitochondrial

localization and co-immunoprecipitation of HADHA/GBP with LC3. A construct consisting of the first 36 residues of HADHA fused to green fluorescent protein (GFP) as reporter (N36-MTS-GFP) allowed to analyse the role of the mitochondrial presequence in determining subcellular localization and secretion. We observed N36-MTS-GFP sorted into mitochondria and also to the medium. Strikingly, both GBP cell surface distribution and N36-MTS-GFP secretion increased upon 24 h of starvation. These results reveal that the presequence of HADHA/GBP contains sorting information to target the mitochondria or the plasma membrane depending on yet unknown regulated processes, in which the cell surface pathway is at least activated by starvation. (Grants: FONDECYT 11181015. CONICYT Basal Project AFB170005).

Calcium and Endoplasmic Reticulum Transport

P1429/B561

Proteomic an alysis Identifies Membrane Proteins Dependent on the ER Membrane Protein Complex.

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The endoplasmic reticulum (ER) membrane protein complex (EMC) is a key contributor to biogenesis and membrane integration of transmembrane proteins, but our understanding of its mechanisms and the range of EMC-dependent proteins remains incomplete. Here we carried out an unbiased mass spectrometry (MS)-based quantitative proteomic analysis comparing membrane proteins in EMC-deficient cells versus wild-type (WT) cells, and identified 36 EMC-dependent membrane proteins and 171 EMC-independent membrane proteins. Of these, six EMC-dependent and six EMC-independent proteins were further independently validated. We found that a common feature among EMC-dependent proteins is that they contain transmembrane domains (TMDs) with polar/charged residues. Mutagenesis studies demonstrate that EMC-dependency can be converted in cells by removing or introducing polar/charged residues within TMDs. Our studies expand the list of validated EMC-dependent and -independent proteins, and suggest that EMC is involved in handling TMDs with residues challenging for membrane integration.

P1430/B562

TRPM2-mediated Ca^{2+} Entry Promotes Neurotoxin-induced Oxidative Stress, Mitochondrial Fragmentation and Neuronal Cell Death.

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The Transient receptor potential melastatin 2 (TRPM2) is a Ca^{2+} permeable ion channel. It is activated during oxidative stress, affecting cellular homeostasis of both Ca^{2+} and Zn^{2+}. TRPM2 is associated with a number of degenerative diseases including diabetes, Alzheimer's and Parkinson's diseases (PD). A defining feature of these diseases is a rise in the intracellular levels of reactive oxygen species (ROS) and increased mitochondrial fragmentation. We have previously demonstrated that TRPM2 activation underlies mitochondrial fragmentation and pancreatic β-cell death. In this study, we asked whether TRPM2 channels play a similar role in neuronal cell death seen in PD, and whether TRPM2 channels regulate ROS, a key driver of mitochondrial fission. To address these questions, we have used the neurotoxin, 1-methy-4-phenylpyridinium (MPP^+) to induce PD-associated changes in the SH-SY5Y neuroblastoma cell line. We have used fluorescent probes to detect changes in ROS (total and

mitochondrial) production, intracellular distribution of Ca^{2+} and Zn^{2+}, mitochondrial morphology, and cell death. Chemical inhibitors of NADPH oxidase (NOX) and TRPM2, and Ca^{2+} and Zn^{2+} chelators, were used to determine the mechanistic role of TRPM2 channels in MPP^+-induced cellular events. We found MPP^+ to induce Ca^{2+} entry, leading to NOX activation and that NOX-derived ROS, in turn, triggered mitochondrial ROS production. Notably, MPP^+ caused a rise in mitochondrial Zn^{2+}, suggesting that rise in mitochondrial Zn^{2+} could stimulate ROS generation by inhibiting mitochondrial electron transport. TRPM2 inhibition attenuated MPP^+-induced Ca^{2+} influx, and the subsequent rise in mitochondrial Zn^{2+}, ROS production, as well as mitochondrial fission and cell death. Inhibition of rise in mitochondrial Zn^{2+} with TPEN, a Zn^{2+} chelator, attenuated ROS production, mitochondrial fission and neuronal cell death. To determine the potential interplay between Ca^{2+} and Zn^{2+}, we used ionophores in conjunction with chelators. A23187-induced rise in cytosolic Ca^{2+} triggered mitochondrial fission and cell death; these changes were prevented by Zn^{2+} chelation, indicating a critical role for Zn^{2+}downstream of Ca^{2+}. Rising mitochondrial Zn^{2+} with the Zn^{2+}-ionophore, pyrithione, alone was sufficient to cause mitochondrial ROS generation, fragmentation and cell death. Taken together, our results suggest that TRPM2 channels play a key role in upregulating ROS production and subsequent neuronal cell death by causing Ca^{2+}-induced rise in mitochondrial Zn^{2+} and subsequent mitochondrial fragmentation.

P1431/B563

Live Imaging in Liver Cells to Study Virus-ca^{2+} Interactions and ER-mitochondria Junctions-regulated Ca^{2+} Homeostasis.

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Hepatitis B virus (HBV) is the leading cause of hepatocellular carcinoma (HCC), but how HBV induces HCC remains unknown. HBx, one of the HBV proteins, was reported to be responsible for HCC initiation, and that oncogenic effect of HBx might require Ca^{2+} signaling in hepatocytes. Recent study also implied that HBx disturbed Ca^{2+} signaling in HCC cells. However, the mechanistic link between HBx, Ca^{2+} signaling and HCC initiation remain elusive. We therefore using live-cell Ca^{2+} imaging to explore how HBx affected Ca^{2+} signaling, and how Ca^{2+} affected the tumorigenic effect of HBx in HCC cells. We established a real-time Ca^{2+} monitoring platforms, which can simultaneously demonstrate local Ca^{2+} dynamics in cytosol, mitochondria and/or endoplasmic reticulum (ER), by stably expressed genetic-encoded Ca^{2+} indicators (GECIs) in HCC and primary hepatocyte cell lines. Assays to measure Ca^{2+} flow between organelles were also designed to elucidate the Ca^{2+} dynamics between those compartments. Using this platform we first discovered that (1) HBx may not directly disturb the intracellular Ca^{2+} dynamics in hepatocytes, but HBx effects on hepatocyte signaling require proper Ca^{2+} dynamics, (2) There exist distinct statuses of mitochondrial Ca^{2+} dynamics, and (3) Local Ca^{2+} oscillations occur in the mitochondria. These findings about mitochondrial Ca^{2+} may be belated to the connectivity statues of ER and mitochondria. To further elucidate the role of ER-mitochondria junctions (EMJs) in whole cell Ca^{2+} homeostasis, we break EMJs by knocking down the tethering proteins, mitofusin2 (MFN2) and PDZD8 in our platform. Indeed, EMJ breakdown increased Ca^{2+} stores in ER and reduced mitochondria Ca^{2+} levels. Surprisingly, knockdown of MFN2 or PDZD8 also suppressed store-operated Ca^{2+} entry (SOCE). Double knockdown of the EMJ tethering proteins MFN2 or PDZD8 and the SOCE components STIM1 and/or ORAI1 eliminated the effects of MFN2 or PDZD8 knockdown. Thus, mitochondria may contribute to STIM1/ORAI1-mediated SOCE via EMJs. We are currently investigating the molecular mechanisms and physiological/pathological

significance of the above phenomena, which will not only revolutionize our understanding of virus-Ca^{2+} interactions and mitochondrial Ca^{2+}-SOCE regulation, but also inspire our potential to develop novel strategies against HBV-induced HCC.

P1432/B564

Alteration in Intracellular Ca^{2+} Levels through Ion Chennels in Human Endometrial Stromal Cells.

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Calcium (Ca^{2+}) is an important element for many physiological functions of the uterus, including embryo implantation. Here, we investigated the possible involvement of altered intracellular Ca^{2+} levels in decidualization in human endometrial stromal cells (hEMSCs). hEMSCs showed high levels of mesenchymal stem cell marker expression (CD73, CD90, and CD105) and did not express markers of hematopoietic progenitor cells (CD31, CD34, CD45, and HLA-DR). Decidualization is a process of ovarian steroid-induced endometrial stromal cell proliferation and differentiation. Several types of ion channels, which are regulated by the ovarian hormones progesterone and estradiol, as well as growth factors, are important for endometrial receptivity and embryo implantation. The combined application of progesterone (1 μM medroxyprogesterone acetate) and cyclic AMP (0.5 mM) for 6 days not only elevated inositol 1,4,5-triphosphate receptor (IP_3R)-mediated Ca^{2+} release and *IP_3R* expression, it also promoted *ORAI* and *STIM* expression as well as cyclopiazonic acid-induced Ca^{2+} release. Finally, intracellular Ca^{2+} levels and ion channel gene expression influenced hEMSC proliferation. These results suggest that cytosolic Ca^{2+} dynamics, mediated by specific ion channels, serve as an important step in the decidualization of hEMSCs.

P1433/B565

The Ca^{2+} Channel TRPML3 Regulates Autophagy by Interaction with VTI1B and ATG16L1.

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TRPML3 is a Ca^{2+}-permeable cation channel that is expressed in multiple subcellular compartments, including the plasma membrane, endocytosis and autophagy pathways. We have previously shown that upon induction of autophagy, TRPML3 traffics to the phagophore by palmitoylation and Ca^{2+} release via TRPML3 is crucial for autophagosome formation. However, it is still not clear from which compartment TRPML3 originates to function in autophagy. To search interacting partners with TRPML3 in autophagy, we performed split-ubiquitin membrane yeast two-hybrid screening and identified VTI1B, a SNARE protien that is required for ATG16L1 precursor homotypic fusion. Since ATG16L1 vesicles are formed by endocytosis and associated into the phagophore, we hypothesized that TRPML3 may move along the same path with ATG16L1 by interaction with VTI1B. Indeed, TRPML3 was also internalized upon autophagy stimulation by mTOR inhibition, while accumulated at the plasma membrane by mTOR activation. Inhibition of TRPML3-VTI1B interaction altered TRPML3 internalization, leading to increased surface expression. Interestingly, the retention of TRPML3 at the plasma membrane caused by any reason was accompanied by suppressed autophagy, suggesting that like in ATG16L1, proper endocytosis of TRPML3 is necessary for autophagy. We found that TRPML3 not only localized in ATG16L1-positive vesicles but also interacted with ATG16L1. Moreover, the interaction between TRPML3 and ATG16L1 was increased by autophagy stimulation but decreased by autophagy suppression. Collectively, our data

suggest that the Ca^{2+} channel TRPML3 is recruited from the plasma membrane to the early autophagic structures by interaction with VTI1B and ATG16L1 to regulate autophagy.

P1434/B566

Orp3 Phosphorylation Regulates Phosphatidylinositol 4-phosphate and Ca^{2+}Dynamics at Plasma Membrane - Endoplasmic Reticulum Contact Sites.

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Oxysterol-binding protein (OSBP)-related proteins (ORPs) mediate non-vesicular lipid-transfer between intracellular membranes. Previous studies revealed the role of phosphoinositide (PPIn) gradients in the ability of OSBP and some ORPs to transfer cholesterol and phosphatidylserine (PS) between the ER and other organelle membranes. Our recent work focused on a less characterized ORP family member, ORP3, as a potential regulator of lipid homeostasis at contact sites between the plasma membrane (PM) and the ER. ORP3 associates with the ER via interaction with vesicle-associated membrane protein-associated proteins (VAPs) and binds to the PM only after activation of protein kinase C (PKC). However, it remains unclear what lipids are specifically transported by ORP3 and what determines its PM localization. Previous studies have suggested that 3-phosphorylated PPIns are recognized by the isolated pleckstrin homology domain of ORP3. Here we usedGFP-tagged forms of ORP3 to investigate in living cells whether PM PPIns contribute to the interaction of ORP3 with the PM. A chemically-inducible heterodimerization approach was used to selectively reduce PM PPIns levels by recruiting PPIn phosphatases directed against either the 4- or 5-phosphate of phosphatidylinositol 4-phosphate (PI4P) and/or phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) to the PM. PM attachment of ORP3 was monitored with TIRF microscopy, while changes in PPIn lipids were measured by BRET-based biosensors in living cells. Our results showed that ORP3 PM association was mainly determined by $PI(4,5)P_2$and to a lesser degree, by PI4P levels of the PM. Furthermore, full activation of ORP3 achieved by a combination of PMA and thapsigargin treatment, robustly decreased PM PI4P levels. Acute artificial establishment of PM association of ORP3 using the chemically-inducible heterodimerization approachled to a small reduction in PM phosphatidic acid (PA) levels and a slightly increase in PM cholesterol level in addition to the massive depletion of PM PI4P. Lastly, ORP3 activation inhibited Ca^{2+}entry via the store-operated Ca^{2+}entry pathway by a mechanism that required the C-terminal segment of the ORP3 protein.

P1435/B567

Store-operated Ca^{2+} Channels Are Involved in Erythropoiesis.

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Erythropoiesis is the process which produces red blood cells from erythropoietic stem cells. Ca^{2+} is a fundamental second messenger which regulates dynamic cellular processes, such as, cell differentiation, proliferation, cytoskeletal rearrangement, and cell cycle. Ca^{2+} signaling is also known to involve in erythropoiesis. However, underlying mechanisms and specific function of Ca^{2+} channels in erythropoiesis remain elusive. Here, we identified the effects of Store-Operated Ca^{2+} channels (SOCs) inhibitors in erythroid lineage determination and proliferation of CD34+ hematopoietic stem cells (HSCs) with colony-forming unit (CFU) assay. SOCs inhibitors reduced the proportion of erythroid lineage and proliferation rate. Next, we demonstrated that the mRNA expression level of SOCs modulators, STIM1

and STIM2, increased during erythropoiesis. Besides, we observed two different patterns of cytosolic Ca^{2+} fluctuation in the maturation stage of RBC formation by doing Fluo-4 Ca^{2+} imaging. In conclusion, this study suggests a novel role of SOCs during erythropoiesis, red blood cell formation.

P1436/B568

The Intramolcular Interaction of IDstim within STIM1 Prevents a Spontaneous Ca^{2+} Entry.

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SOCE (store-operated calcium entry) is a major calcium ion influx pathway and abnormal SOCE activation causes severe diseases such as Stormorken syndrome, York platelet syndrome, and tubular aggregate myopathy. To control SOCE, STIM1 undergoes a conformational change in its cytosolic domains. STIM1 was known to maintain an inactive state through hydrophobic interaction between CC1 (coiled-coil domain 1) and CAD (CRAC activating domain). However, the CC1-CAD fragment showed constitutive activation despite maintaining the hydrophobic interaction. This conflicting observation implies that an additional mechanism is required to maintain the inactive state of STIM1. Here, we show that IDstim (inactivation domain of STIM1) binds to and inhibits CC1-CAD and this inhibitory effect of IDstim is abolished by either CC1α1 deletion or leucine substitution. The conserved short linker between CC1-CAD and IDstim facilitates the IDstim-mediated STIM1 inhibition. Our findings expand our understanding how the unintended activation of SOCE is prevented under resting conditions.

P1437/B569

***Disease-associated Mutations in Niemann-Pick Type C1 Alter ER*Ca^{2+}*and IP_3R Mediated Signaling Pathways*.**

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NPC1 is a transmembrane lysosomal protein involved in transporting free cholesterol from lysosomes to other cellular membranes, including the endoplasmic reticulum (ER). This transfer of cholesterol to the ER regulates the activity of the sterol-response element binding protein (SREBP), a transcription factor governing the genes involved in cholesterol homeostasis. The importance of cholesterol regulation by NPC1 and SREBP is underscored by the fatal neurodegenerative disease: Niemann Pick Type C1 (NPC1 disease), where loss of function mutations in NPC1 lead to significant accumulation of free cholesterol within lysosomes and activation of the SREBP pathway due to the loss of cholesterol transfer to the ER. We have determined that in NPC1 disease, ER Ca^{2+} signaling pathways are significantly impaired with patient cells having reduced luminal ER Ca^{2+}, potentiated IP_3R Ca^{2+} release, and enhanced store-operated calcium entry (SOCE) compared to healthy controls. A combination of super-resolution imaging, qPCR, western blot, and pharmacology, reveals that SREBP is upstream of many expressional changes of Ca^{2+} handling proteins in NPC1 disease, including an increase in expression of presenilin 1 (PS1); a purported Ca^{2+} leak channel at the ER. Using a genetically encoded Ca^{2+} sensor targeted to the ER lumen we have measured a significant increase in ER Ca^{2+} leak in NPC1 disease. Treating NPC1 disease fibroblasts with siRNA targeting PS1 rescues this ER Ca^{2+} phenotype. Moreover, an inhibitor of NPC1 (U18666A), which recapitulates the Ca^{2+} phenotypes of NPC1 disease in multiple cell types, elicits no Ca^{2+} change in $PS1^{-/-}$ cells. Treatment of NPC1 disease fibroblasts with SREBP inhibitor, PF-429242, rescues PS1 expression as well as ER Ca^{2+} and SOCE, suggesting a close relationship between SREBP and

PS1. Given PS1 has been proposed to interact with IP_3R to regulate IP_3R-Ca^{2+} signaling in neurons, we investigated if GqPCR Ca^{2+} signaling is altered in NPC1 disease. Western blot analysis, super-resolution microscopy and Ca^{2+} imaging revealed a 3-fold increase in IP_3R protein with increases in puncta size, density, and IP_3R mediated Ca^{2+} release. Overexpression of the PS1 catalytically-dead holoprotein (PS1-D257A) results in larger clustering of IP_3R and enhanced Ca^{2+} release following activation of Gq-coupled receptors. Conversely, overexpression of the wild-type form of PS1 does not alter IP_3R-mediated Ca^{2+} release, suggesting the holoprotein form of PS1 specifically mediates these cellular changes. Together, these data suggest that the holoprotein form of PS1 at the ER is able to decrease ER Ca^{2+} levels leading to constitutive SOCE, and promote clustering of IP_3R to augment Gq-coupled receptor activation.

P1438/B570

The Role of Voltage-gated Ion Channels in Phagocytosis of Photoreceptor Outer Segments.

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Background: Retinal pigment epithelium (RPE), important retina maintenance tissue in the back of the the eye, has a critical role in photoreceptor renewal where phagocytosis of photoreceptor outer segments (POSs) occurs in a diurnally synchronized process. This process is precisely regulated, however, the regulation is not yet completely understood. Earlier work on macrophages have raised the question about the involvement of ion channels in phagocytosis. Interestingly, changes in intracellular Ca^{2+} concentration have been indicated to affect the phagocytic activity of RPE. In addition, recent work suggests the contribution of Ca^{2+}-dependent ion channels to POS phagocytosis. Despite the existing knowledge, we have only a limited understanding on the influence of ion channels on phagocytosis. Our aim was to investigate the role of voltage-gated Ca^{2+} (Ca_v) and Na^+ (Na_v) channels that we have recently identified in RPE, to POS phagocytosis. Methods: the work was performed on human embryonic stem cell-derived RPE (hESC-RPE) and mouse RPE. Ion channel expression was studied by mass spectrometry analysis and functionality by whole-cell patch clamp recordings. Ion channel localization was determined by immunostainings and immunogold electron microscopy (EM). Phagocytosis was assayed by feeding porcine POS to RPE and incubating the samples either at room temperature (binding phase) or at 37°C (processing phase) with or without the pharmacological ion channel modulators. Results: Our patch clamp recordings showed the presence of functional L- and T-type Ca_v channels together with several Na_v channel subtypes in RPE. These channels participated in POS phagocytosis: pharmacological modulation of their activity affected phagocytosis by increasing or decreasing the amount of bound and ingested POS particles, depending on the modulator. Specifically, inhibition of Na_v channels by pharmacology or shRNA silencing affected phagocytosis by decreasing the amount of ingested POS particles while not affecting their binding. Immunolabeling results showed that in both hESC-derived and mouse RPE, these channels concentrated on the phagosomes, and immuno-EM confirmed this finding. Conclusions: Our results indicate that Na_v and Ca_v channels are an integral part of RPE physiology, and that they contribute to POS phagocytosis. The role of Na_v channels is to co-regulate the processing phase of bound photoreceptor outer segments. Further studies are needed in order to fully evaluate the concerted functioning of these ion channels in phagocytosis. References: Johansson et al. 2019, *BMC Biology* **17**, No: 63; Korkka et al. 2019, *Stem Cells Translational Medicine*. **8**(2):179-193.

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Lipid and Membrane Microdomains 2

P1439/B571

Towards Structure of the Human Spt-ORMDL Sphingolipid Regulatory Complex.

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Towards structure of the human SPT-ORMDL sphingolipid regulatory complex. Muthukumar Kannan[1], Weihua Qiu[2], Youzhong Guo[2 and] Binks Wattenberg[1] [1]Department of Biochemistry and Molecular Biology, Virginia Commonwealth University, Richmond, VA 23298. [2]Department of Medicinal Chemistry, Virginia Commonwealth University, Richmond, VA. **Abstract** Sphingolipids are a group of lipids which are found in abundance in all eukaryotic cell membranes as well as acting as critical signaling molecules. The cellular level of sphingolipid is maintained by the first and rate–limiting enzyme in the *de novo* synthesis pathway, serine palmitoyltranferase (SPT). SPT is a multi-subunit enzyme localized to endoplasmic reticulum (ER) in yeast and mammals. The activity of SPT is homeostatically regulated by another ER resident protein, ORMDL, which is crucial for regulating the *de novo* biosynthesis of ceramide. SPT forms a stable complex with ORMDL to precisely maintain the cellular ceramide level. Increased levels of cellular ceramide inhibits SPT activity in an ORMDL-dependent manner. The mechanism behind the regulation of SPT activity by ORMDLs is largely unknown. To address these critical gaps in our knowledge, currently we are studying the structure of SPT-ORMDL complex. We expressed the human SPT and ORMDL in yeast cells. We believe this will be the excellent tool to understand the structural basis of ORMDL dependent SPT regulation. Our initial experiments suggest that both human SPT and ORMDL are expressed in functionally active form in yeast and in yeast, human ORMDL regulates SPT activity in response to elevated levels of sphingolipids.

P1440/B572

Sphingolipid Biosynthesis during Myelination in the Developing Rat Brain.

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Myelin is a specialized extension of plasma membrane produced by oligodendrocytes in the central nervous system (CNS). It consists of specific lipids and proteins produced in a coordinated process. As compared to other biological membranes, lipid content is very high. Sphingolipids comprise a major portion of myelin lipids. Previous studies have shown that sulfatides, cerebrosides and sphingomyelin are the major constituents of myelin sphingolipids and the timing of their production has also been reported to some extent. Understanding how individual lipid and protein components are regulated to produce optimal lipid composition is essential for understanding the basics of production of this highly specialized membrane and how dysregulation of its production contributes to devastating demyelinating diseases. To address above question, we utilized the well characterized myelination program in neo-natal rats. Rat pups were sacrificed from postnatal day 2 to 30. In addition, we isolated oligodendrocytes, the CNS cell type that produces myelin. Comprehensive sphingolipid profiling was performed in total brain and oligodendrocytes. Using mass spectrometry, we measured the levels of d18:sulfatides (3-O-sulfogalactosylceramide, a sulfated sphingolipid) and found that sulfatide starts rising from day 5 to day 25 where it plateaus. Similarly, levels of d18:monohexosalceramides (cerebrosides) rises at day 9 and plateau at day 25. The d:18ceramide and sphingomyelin levels were

found to be constant throughout the myelination. Next we measured d18:sphingosine and d18:dihydrosphingosine, upstream metabolites in sphingolipid pathway. We found that sphingosine levels rise gradually during myelination process but dihydrosphingosine levels rise more dramatically late in process. We also found that the molecular species of sphingolipids, i.e. The length of the fatty acyl component of sphingolipid, undergo dramatic changes during onset of myelination. Moreover, there were marked increase in the levels of non-canonical sphingolipid backbones, particularly the d:20 species. To gain an understanding of the molecular basis of these changes we measured the protein, mRNA and activity of the rate limiting enzyme complex in sphingolipid biosynthesis, serine palmitoyltranferase (SPT). We found dramatic changes in the composition of the SPT complex, including changes in levels of the regulator of SPT, the ORMDLs. These studies will form the basis for understanding the coordination of the lipid and protein components of myelin and how these might be manipulated for the treatment of demyelinating diseases.

P1441/B573

Mammalian Ormdl Proteins Respond Stereospecifically to Ceramide Species to Regulate *De Novo* Sphingolipid Biosynthesis.

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Sphingolipids are a diverse class of lipids built on a sphingosine backbone which have important functions in cell signaling and membrane structure. Serine palmitoyl transferase (SPT) serves as the initiating and rate-limiting enzyme of the *de novo* biosynthesis of sphingolipids. SPT is homeostatically regulated by a mechanism which requires the ORMDL proteins in mammalian cells and ORM proteins in yeast. ORMDLs and ORMs, which are membrane-bound proteins in the endoplasmic reticulum, function to reduce SPT activity in response to elevated cellular sphingolipid levels. Previous studies have shown that ORMDLs mainly sense ceramide. However, for technical reasons, the ceramides used in those studies had chain lengths that were much shorter than natural ceramides. Here we attempt to recapitulate the inhibition of ORMDL-dependent inhibition of SPT by endogenous ceramides using ceramide synthase (CerS) to generate ceramides with natural chain lengths. CerS uses Acyl-Coenzyme as (Acyl-CoA) and either sphingosine or dihydrosphingosine to generate ceramide or dihydroceramide, respectively. To recapitulate the sphingolipid-dependent regulation of SPT by ORMDLs, we recently developed a cell-free system in which membranes were isolated and used to test SPT activity. To generate ceramides with native chain lengths within those membranes using the activity of the endogenous CerS, we pre-incubated membranes with the 24:1 CoA and sphingosine or dihydrosphingosine either separately or together. We demonstrate that SPT activity is inhibited in an ORMDL-dependent process only under conditions in which ceramide is generated. Moreover, we find that both ceramide and its immediate precursor, dihydroceramide, are sensed by this system. Using the CerS inhibitor, Fumonisin B1, we have established that the ORMDLs do not respond to sphingosine or dihydrosphingosine. Furthermore, we tested the four stereoisomers of ceramide and find that only the native stereoisomer of ceramide is able to trigger SPT inhibition. This strongly suggests that the ORMDL/SPT complex senses ceramide levels by a direct binding interaction. These results establish ORMDL as a negative regulator of SPT that directly senses cellular ceramide levels.

P1442/B574

Seipin Negatively Regulates Sphingolipid Production at the Er-ld Contact Site.

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Seipin is an evolutionarily conserved protein regulating both adipocyte development and lipid droplet (LD) biogenesis. With its evidently crucial roles in controlling LD assembly at the contact site between endoplasmic reticulum (ER) and LDs, the molecular function of seipin has long been a focus in the field, yet remains largely controversial. Here, we identified a new function of seipin as a negative regulator for sphingolipid production. We provide evidence that seipin negatively regulates sphingolipid production by binding with two key enzymes, namely serine palmitoyltransferase and fatty acid elongase, thereby regulating the production of two major building blocks for sphingolipids, termed long chain base and very long chain fatty acid, respectively. We then uncovered that the regulations are organized at discrete regions of the ER in close vicinity to LDs, which led to the hypothesis that the ER and LD contact site is one place in the ER where cells synthesize sphingolipids. To decipher the potential interconnection of sphingolipid synthesis and LD formation at the same subdomain, we provide further evidence that LDs can form efficiently when sphingolipid synthesis is blocked, whereas excess sphingoid intermediates may affect LD morphology. Expression of human seipin rescued the altered sphingolipids in yeast seipin mutants, suggesting that the negative regulation of sphingolipid synthesis by seipin is likely an evolutionarily conserved process.

P1443/B575

Defining the Subcellular Distribution and Metabolic Channeling of Phosphatidylinositol.

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Among the structural phospholipids that form the bulk of eukaryotic cell membranes, phosphatidylinositol (PtdIns) is unique in that it also serves as the common precursor for low-abundance phosphorylated derivatives, called polyphosphoinositides (PPIn), which are central regulators of cellular physiology. In particular, the complex metabolic turnover of PPIn species has essential functions related to both intracellular signal transduction and membrane trafficking, however, there is still a limited understanding of how PtdIns synthesis and transport contributes to the turnover of the unique subcellular pools of PPIn lipids. To address these shortcomings, we established a molecular toolbox for investigations of PtdIns distribution and availability within intact cells that capitalizes on the substrate selectivity and high specific activity of the bacterial PtdIns-specific phospholipase C (bacPI-PLC) enzyme. Results using catalytically inactive mutants of the bacPI-PLC, which were designed to trap the bound PtdIns substrate, suggest that PtdIns is localized to the endoplasmic reticulum (ER), the biochemically-defined site of PtdIns synthesis, but is also enriched in the cytosolic leaflets of the Golgi complex, peroxisomes, and mitochondria. Strikingly, we did not observe significant localization of the bacPI-PLC variants within the plasma membrane (PM) or to endosomal compartments in any of the mammalian cell types examined. The membrane distribution of PtdIns was further investigated using recruitable versions of a modified bacPI-PLC scaffold to map PtdIns contents by monitoring the local production of diacyglycerol (DAG), the direct cleavage product of bacPI-PLC-mediated PtdIns hydrolysis. A chemically-inducible protein heterodimerization system was used to target the bacPI-PLC to specific organelle membranes and revealed the rapid production of DAG within the cytosolic leaflets of the ER,

mitochondria, peroxisomes, and Golgi complex. However, only minor increases in DAG production were observed following the targeted recruitment of the bacPI-PLC to the PM or to Rab5- and Rab7-positive endosomes. The recruitable bacPI-PLC construct was then used for kinetic studies on PPIn turnover that demonstrate the requirement for sustained PtdIns supply from the ER, rather than the absolute steady-state content of PtdIns, for the maintenance of monophosphorylated PPIn species within the PM, Golgi complex, and endosomal compartments. Overall, our findings provide a comprehensive map of the subcellular distribution of PtdIns within the membrane compartments of intact cells as well as support an important role for PtdIns transfer and substrate channeling in the spatial control of PPIn metabolism.

P1444/B576

A High-avidity Biosensor Reveals Plasma Membrane PI(3,4)P_2 is Predominantly a Class I PI3K Signaling Product.

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Class I phosphoinositide 3-OH kinase (PI3K) signaling is central to animal growth and metabolism, and pathological disruption of this pathway affects cancer and diabetes. However, the specific spatial/temporal dynamics and signaling roles of its minor lipid messenger, phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2), are not well understood. This owes principally to a lack of tools to study this scarce lipid. Here we developed a high-sensitivity genetically encoded biosensor for PI(3,4)P2, demonstrating high selectivity and specificity of the sensor for the lipid. We show that despite clear evidence for class II PI3K in PI(3,4)P2-driven function, the overwhelming majority of the lipid accumulates through degradation of class I PI3K-produced PIP3. However, we show that PI(3,4)P2 is also subject to hydrolysis by the tumor suppressor lipid phosphatase PTEN. Collectively, our results show that PI(3,4)P2 is potentially an important driver of class I PI3K-driven signaling and provides powerful new tools to begin to resolve the biological functions of this lipid downstream of class I and II PI3K.

P1445/B577

Control of Circadian Rhythm Via Surface Density of Muscarinic Acetylcholine Receptors and Cytoplasmic Lipid Kinase Activities in Sympathetic Neurons.

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Superior cervical ganglion (SCG) neurons play an important role for controlling the circadian rhythm through innervation of the pineal gland. To further our understanding of this pathway, we analyzed action potential firing and its dependence on the phospholipid phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) in SCG neurons from adult male rats through electrophysiological, biochemical, and mathematical approaches. Previously, we found action potential firing to be controlled by KCNQ2/3 and Maxi-K potassium channels, and a decrease in their activities by 80% was necessary to evoke action potential firing. Physiologically, action potential firing in SCG neurons is induced by hydrolysis of $PI(4,5)P_2$. While KCNQ2/3 channel activity has been shown to be strongly dependent on $PI(4,5)P_2$, a regulation of Maxi-K channel activity by phosphoinositides is under debate and has been speculated to be dependent on the subunit composition of Maxi-K channels. To analyze Maxi-K subunit expression in SCGs, we isolated total RNA from SCGs and performed RT-PCR with oligonucleotides for α- and β-subunits of Maxi-K channels. Our results showed expression of only the Maxi-K α-subunit. Expression of Maxi-K α-subunits in HEK293 cells showed no decrease in Maxi-K channel activity upon depletion of

$PI(4,5)P_2$ by either activation of muscarinic acetylcholine receptors or a voltage-sensitive lipid phosphatase, thereby identifying KCNQ2/3 channels as the sole $PI(4,5)P_2$-dependent potassium channels in SCG neurons. We incorporated our electrophysiological and biochemical results into a model of phosphoinositide metabolism of SCG neurons to develop a mathematical description of phosphoinositide metabolism and $PI(4,5)P_2$-dependent ion channel activity in SCG neurons. The model allowed for reproduction of experimentally determined characteristics of action potential firing with respect to phosphoinositide-dependent ion channel activities in SCG neurons, and highlighted critical roles for the control of cellular excitability by the density of muscarinic acetylcholine receptors and levels of $PI(4,5)P_2$ through cytoplasmic concentrations of lipid kinases. In conclusion, the newly developed model allows for the first time a simulation of phosphoinositide metabolism and its regulation of action potential firing of SCG neurons through modulation of muscarinic acetylcholine receptor activity, thereby providing an opportunity to analyze modulation of circadian activity in *silico*.
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P1446/B578

Comparison of Various Energy Transfer-based Biosensors to Monitor Phosphatidylinositol-4,5-bisphosphate Levels in the Plasma Membrane of Mammalian Cells.

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Phosphatidylinositol-4,5-bisphosphate (PIP2) is found in the plasma membrane (PM) and plays an important role in the molecular signal transduction pathway initiated by Gq protein-coupled receptors, such as the type-1 angiotensin receptor (AT1R). Our research uses a rapamycin-induced translocation of an FKBP-fused 5-phosphatase (FKBP-5ptase) or stimulation of AT1R to modify and measure PM PIP2 levels using various phosphatidylinositol biosensors. Bioluminescence resonance energy transfer (BRET)-based biosensors were created and applied to determine which of them is most sensitive to PM PIP2 level changes. A modified type-1 angiotensin receptor (AT1R-Δ319), FKBP-5ptase and various phosphoinositide biosensors were transiently expressed in HEK 293 cells. BRET measurements were carried out 27-28 hours later. PIP2 depletion was achieved upon stimulation with angiotensin II or rapamycin-evoked translocation of the FKBP-5ptase to the PM. The biosensors were created from known lipid binding domains, such as the PH domains of PLCδ1 and PLCδ4 and the C-terminal part of Tubby (Tubby-C). In addition to the original PIP2 sensor (PLCδ1-PH), our study used the following sensors: PLCδ4-PH, Tubby-C, Tubby-C with a K330A mutation, and their tandem versions (PLCδ4-2xPH, Tubby-2xC, Tubby-2xC KA). Our results show that PM recruitment of FKBP-5ptase induced an acute depletion of PM PIP2. Sensors with PLCδ1-PH, PLCδ4-PH and Tubby-C domain were all able to follow the decrease of PM PIP2 level, but the tandem of PLCδ4-PH produced a significantly greater signal drop. Surprisingly, the tandem of Tubby domain revealed less response than Tubby-C. Mutated versions of the Tubby domain showed almost no change. Similar results were achieved after stimulation by angiotensin II with the exception of PLCδ4-PH which reflected an increase of the recruitment of the sensor upon hormonal stimulation. Overexpression of FKBP-5ptase showed a significant depletion of PM PIP2 levels

and PLCδ4-2xPH was found to be the most sensitive of the biosensors. Improvement of phosphoinositide biosensors is of great importance for future studies so that they can give information that is more accurate on phosphoinositide levels and lead to a better understanding of their dynamics.

P1447/B579

Stim1 Associates with Vap B and Regulates Calcium Dynamics.

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The store-operated Ca^{2+}-entry (SOCE) pathway is integral to the physiology and function of numerous cell types including neurons and immune cells. In many immune cells, the Ca^{2+}sensor protein STIM1 is localized throughout the endoplasmic reticulum (ER) upon basal resting conditions. Depletion of Ca^{2+}ER stores by FcεRI signaling or the SERCA inhibitor thapsigargin induces STIM1 translocation to the cortical ER where it couples to and activates the Ca^{2+}channel protein Orai1 at the plasma membrane (PM). We find that the ER-localized VAP-B protein associates with the STIM1 protein during SOCE in RBL mast cells, as monitored by fluorescence resonance energy transfer (FRET). A dominant negative form of VAP-B (P56S) implicated in amyotrophic lateral sclerosis (ALS) is impaired in this interaction. Moreover, association of STIM1 with Orai1 is reduced by expression of the P56S mutant protein, in parallel with the partial inhibition of SOCE. Likewise, antigen-induced Ca^{2+}uptake into mitochondria is impaired in cells expressing the mutant VAP-B (P56S) protein. Consistent with these results, siRNA knockdown of the VAP-A and VAP-B isoforms results in impaired SOCE as well as mitochondrial Ca^{2+}uptake stimulated by antigen or thapsigargin. Altogether, our results indicate that the ER-localized VAP-B protein is involved in SOCE and inter-organelle Ca^{2+}handling.

P1448/B580

Endoplasmic Reticulum Stress Sensor Ire1 Deploys a Divergent Transcriptional Programme in Response to Lipid Bilayer Stress.

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The unfolded protein response (UPR), a complex adaptive stress response of the endoplasmic reticulum (ER), is compromised in disease and aging. Typically activated by the accumulation of misfolded proteins within the ER lumen, the UPR is similarly activated by alterations of fatty acids and lipid composition at the ER. Chronic UPR activation by ER lipid aberrations, which we termed lipid bilayer stress-induced UPR (UPR^{LBS}) is associated to the development of the metabolic syndromes. However, most studies to dissect the UPR^{LBS} mechanisms employ exogenous lipids or omit precursors of lipid biosynthesis. To systematically understand pathways that contribute to UPR activation, we performed a genome-wide genetic screen in *S. cerevisiae* and an RNAi screen in *C. elegans* to identify mutations that activate the UPR though LBS. Several unexpected cellular perturbations were identified to induce the UPR through LBS. As one of the strongest hits inducing the UPR, we further characterise the activation mechanism of Ire1 in Δ*opi3* where lack of phosphatidylcholine (PC) synthesis induces LBS. We found that the luminal domain (LD) of Ire1, which senses misfolded peptides, is dispensable to activate the UPR by LBS while LD overexpression was sufficient to uncoupled LBS-induced to proteotoxic-induced UPR. Conversely, we mutated Ire1 residue R537 located at the interface of the amphipathic and transmembrane helices

rendering it insensitive to LBS while retaining the capacity to activate the UPR by proteotoxic stress. Furthermore, transcriptomic and ChIP-qPCR data revealed that the UPR programme diverges if activated by LBS or proteotoxic stress. Together, our data point to the UPR as a broad-spectrum compensatory pathway in which LBS and proteotoxic stress-induced UPR deploy divergent transcriptional programmes.

P1449/B581

Differences in Membrane Mobility between Salivary Gland Cells in Live Mice and Ex Vivo Cultured Tissue.

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Membrane mobility depends on the lateral diffusion of molecules within the lipid bilayer, which reflects their non-covalent interactions between lipids, cholesterols, membrane proteins and macromolecular complexes interacting with the membranes intra- and extra-cellularly (i.e., extracellular matrix, cytoskeleton). Membrane mobility has been primarily assayed in cells cultured in *vitro*. However, these model systems do not always recapitulate the complex organization of membranes in *vivo*. Here we investigate how differences in membrane mobility between membranes in live animals and *ex vivo* model systems affect membrane remodeling dynamics, in particular during endocytosis and exocytosis. To measure membrane mobility in secretory epithelial cells in the salivary glands of live mice, we used fluorescence recovery after photobleaching (FRAP) in genetically engineered mice ubiquitously expressing a membrane-targeted peptide fused wit the fluorescent protein td-Tomato (mTomato). In parallel, FRAP was also performed in mouse salivary glands were explanted, dissected into lobules, and then cultured. We found that membrane mobility of cells in *vivo* was significantly lower than ex vivo cells. Notably, the uptake of selected molecules by salivary secretory epithelial cells in *vivo* was significantly slower than in *in vitro*. In addition, the number of endocytic vesicles in cells in *vivo* was significantly diminished in comparison to the in *vitro* model. We concluded that differences in the membrane mobilities in secretory epithelial cells in mice salivary glands in *vivo* and in *vitro* could potentially regulate the rate of endocytosis.

P1450/B582

***In-vitro* FRET an alysis of Growth Arrest-Specific Protein 7 (GAS7).**

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Many cellular processes that are correlated with membrane remodeling involve the actions of the Bin-Amphiphysin-Rvs167 (BAR) domain proteins. Growth Arrest-Specific protein 7 (GAS7) is one of the FCH (F)-BAR subfamilies. We have recently revealed that GAS7 binds to the lipid membrane and also takes a sheet-like assembly in the crystals, on the reconstituted membrane, and in mammalian cells. GAS7b also is thought to play a role in the formation of phagocytic cup. However, the kinetics of GAS7b oligomerization that assembles into the tightly packed sheet on the lipid membrane had not been revealed. To study the GAS7b assembly into the sheet on membranes, we examined the Fluorescence Resonance Energy Transfer (FRET) that occurred by the interaction between the fluorescent proteins at their vicinities. We tagged cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP) to GAS7b or to GAS7b mutant, which has a mutation that affects the stabilization of GAS7 F-BAR domain, and

observed their interaction on liposome membrane by FRET. Interestingly, we observed the FRET between CFP-GAS7b and YFP-GAS7b only in the presence of liposomes. We also succeeded to observe the time-dependent increase in the FRET of GAS7b, which indicated the rapid oligomerization of GAS7. In contrast, the mutant had significantly lower FRET compared with that of GAS7b. Remarkably, the mutant had different or defective oligomer formation in crosslinking assay, indicating that the position of the mutation was essential for the oligomer assembly through the stabilization of F-BAR and interaction with another dimer of GAS7. Taken together, these results support that GAS7b can form oligomers on the membrane, which will help phagocytic cup assembly. Thus, what GAS7 can do in helping the phagocytosis system to act against the pathogen, as it has role in the phagocytotic cup formation, could be important for the future investigation.

P1451/B583

Structural organization of caveolin-1 8S oligomers determined by cryo-electron microscopy.

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Cholesterol-rich membrane invaginations known as caveolae play a critical role in membrane buffering, mechanotransduction, and cellular signaling. The integral membrane protein caveolin-1 (Cav1) is essential for caveolae formation in eukaryotic cells and can even drive the formation of caveolae-like structures in bacteria. Cav1 contains an unusual hairpin shaped intramembrane domain predicted to bend caveolar membranes by a wedging mechanism and oligomerizes to form 8S complexes that function as the fundamental building blocks of caveolae. However, the molecular architecture of these complexes is currently unknown. To fill this gap in knowledge, we used negative staining and single particle cryo-electron microscopy to determine the structure of Cav1 8S oligomers. By negative staining we show that 8S complexes forms discs that are flat on one side, contain a central protrusion on the other, and vary in diameter. The diameters differ by a fixed amount, suggesting that they contain different numbers of Cav1 monomers. Using Venus as a fiduciary marker, we show the flat side of the disc corresponds to the membrane-facing side of the protein and the C-terminus of Cav1 is localized to the central protrusion. We also generated a ~10 Å resolution structure of the 8S Cav1 complexes using cryo-electron microscopy. At this resolution the discs are reminiscent of a wheel, consisting of an outer ring connected by spokes to an inner protruding ring. Interestingly, the intramembrane domains of Cav1 monomers are localized to the outermost portion of the wheel. Current work is focused on improving the resolution of 8S complex structure in order to build a more detailed molecular model of the complex and integrating a variety of biochemical, biophysical, and structural techniques to dissect how the 8S complexes form. Ultimately, these studies should provide a structural framework for understanding how caveolae assemble and control cellular functions.

P1452/B584

Importance of Lipid Phase State in a Selective Binding of *Bacillus Thuringiensis* Cyt2aa2 Toxin on Lipid Membranes.

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Bacillus thuringiensis (*Bt*) is a well-known bacterium because of its pesticidal property. Cytolytic toxin (Cyt) is one of *Bt* pesticidal proteins produced during its sporulation phase. Cyt2Aa2 toxin exerts its activity against insects and mammalian cells. The toxin directly binds to the lipid membrane and disturbs the membrane integrity. A biological lipid membrane comprises of many type of lipids which are able to form different lipid phase states. Recently, the combination of Atomic Force Microscope (AFM) and Quartz Crystal Microbalance with Dissipation (QCM-D) has been used to investigate the protein-lipid bilayer interaction. In this work, we have investigated the interaction of Cyt2Aa2 toxin with lipid bilayers that presented different lipid phases. Membrane lipids found in the cell membrane were selected to build the supported lipid membranes. The lipid bilayers were formed on silica surfaces by means of lipid vesicle fusion. QCM-D results revealed that Cyt2Aa2 toxin bound faster on L_o bilayers than L_d bilayers by increasing of cholesterol content in the POPC bilayer. However, the 1:1 DPPC/cholesterol ratio produced an exceptional L_o bilayer for Cyt2Aa2 toxin binding. In addition, Cyt2Aa2 toxin did not show any binding to gel phase (S_o) domains of either DPPC or sphingomyelin. Surprisingly, different molar ratios of DPPC/POPC mixture influenced the location for Cyt2Aa2 toxin binding. The L_d domains and L_d-S_o domain interface were the binding area of Cyt2Aa2 toxin on 1:1 and 4:1 DPPC/POPC bilayers, respectively. Furthermore, the specific binding of lipid phase and hemolytic activity relevance was demonstrated for the less active T144A mutant. Both QCM-D and AFM results confirmed the inability of POPC bilayer-T144A mutant binding. Addition of cholesterol and/or sphingomyelin to POPC bilayer (forming L_o bilayers) led to Cyt2Aa2 T144A-lipid binding, but the binding rate remained slower than the wild type. Finally, the Cyt2Aa2-lipid interaction was determined with an erythrocyte cell membrane. With sequential toxin exposure, the T144A mutant initially revealed the L_o domains and the L_d domains were detected later by Cyt2Aa2 wild type indicating to the L_d-L_o phase coexistence in the cell membrane. These findings suggest that L_d and L_o membranes are suitable for Cyt2Aa2 binding whereas S_o and DPPC membranes are unfavorable for the binding.

P1453/B585

Super-resolution Microscopy of the an giotensin Receptor Clustering in the Plasma Membrane.

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The cardiovascular physiology is highly affected by the an giotensin II Receptor type 1 (AT1R) membrane protein. The receptor directs the cardiac cells onto two pathways affecting either muscle contraction or proliferation of cells depending on the physiological context. The pathways can be distinguished by modified an giotensin II analogue which only activate a single pathway. We here show for the first time imaging data directly indicating that nanodomains affects the AT1R signal transduction. Therefore, knowledge of receptor dynamics in the plasma membrane and nanodomain formation in the different pathway could sheet light on the regulation mechanism. Photoactivated localization microscopy (PALM)

and pathway specific agonist allow us to identify the role of nanodomains in the regulation of the pathways. Preliminary data based on single particle tracking and k-space image correlation spectroscopy reveals differences in diffusion behavior depending on the stimulation, indicating pathway selection depends on AT1R nano-localization. Our results provide the first look at the broad, nanoscale organization and dynamics of AT1R, opening the door for a deeper understanding of AT1R regulation.

P1454/B586

Synthetic Membranes for the Reconstruction of Cellular Functions from the Bottom Up.

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The reconstruction of the functions of living cells using non-natural components is one of the great challenges of natural sciences. Compartmentalization, encapsulation and surface decoration of globular assemblies, known as vesicles, represent key early steps in the reconstitution of synthetic cells. During my time at University of Pennsylvania I demonstrated that vesicles self-assembled from amphiphilic Janus dendrimers, called dendrimersomes, encapsulate high concentrations of hydrophobic components and do so more efficiently than commercially available stealth liposomes assembled from phospholipid components. These multi-layer onion-like dendrimersomes demonstrate a particularly high capacity for loading low molecular weight compounds and even folded proteins. Co-assembly of amphiphilic Janus dendrimers with metal-chelating ligands conjugated to amphiphilic Janus dendrimers generates dendrimersomes that selectively display folded proteins on their periphery in an oriented manner. A modular strategy for tethering nucleic acids to the surface of dendrimersomes is also demonstrated. These findings augment the functional capabilities of dendrimersomes to serve as versatile biological membrane mimics for the devise of synthetic cells and represent an useful strategy for the delivery of hydrophobic drugs.

P1455/B587

Effect of Hydrophobic Small Molecules on the Lipid Bilayer in the Presence of Cholesterol: What Can Differential Scanning Calorimetry Tell Us?

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In cell biology, the thermal phase transitions of lipids within cell membranes can be characterized using differential scanning calorimetry (DSC). Understanding the thermotropic phase behavior when small hydrophobic molecules are incorporated into the hydrophobic region of membranes may have implications for in *vitro* studies where hydrophobic compounds are introduced into cells, and studies involving the incorporation of hydrophobic drugs in drug delivery systems. Phosphatidylcholine lipids when dispersed in an aqueous milieu form bilayer membrane structures known as liposomes that are used as simplified cell membrane mimics. When small molecules are added to these vesicles there is an effect on the thermal transitions of the lipids as has been classically noted for the addition of cholesterol. Previous research suggests that the hydrophobicity of small molecules added to a membrane have a profound effect on the thermal transitions implying that these molecules sit at different depths in the membrane bilayer. The resulting shifts in melting transition have been characterized as lower onset temperatures and lower enthalpies of melting. To our knowledge, studies have not been done to combine the effects of hydrophobic small molecules with cholesterol in the

membrane. In our research, DSC was used to characterize the phase transitions in dipalmitoylphosphatidylcholine (DPPC) liposomes in the presence of a hydrophobic small molecule, N-phenyl-1-naphthylamine (NPN). Three different preparations were added to DPPC liposomes: NPN only (added using dimethyl sulfoxide (DMSO)), cholesterol only, and both NPN and cholesterol. The presence of NPN alone lowers the temperature and enthalpy of melting resulting in a decrease of the onset and peak temperatures by 0.896°C and 0.417°C, respectively in comparison to the mock treated DPPC control. The enthalpy, however, changed less than 0.200 J/g between the NPN sample and the mock treated DPPC control. In the presence of cholesterol alone, the onset and peak temperatures decreased 1.08°C and 1.04°C in comparison to mock treated DPPC control. The enthalpy for cholesterol only also changed less than 0.200 J/g from the control. In the presence of both NPN and cholesterol, the onset and peak temperatures decreased 1.93°C and 1.54°C in comparison to mock treated DPPC control. The most significant of these findings is for the liposomes with both cholesterol and NPN incorporated, with a more than 0.800 J/g difference in enthalpy from the control. Interestingly, this phenomenon does not follow the trend from the small molecules incorporated individually. Research is ongoing to vary the amounts of the incorporated molecules to elucidate a trend in these values.

P1456/B588

INPP5E Is Required for Recruitment of the TCR/CD3 Complex to the Immune Synapse Via the Ciliary Machinery.

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The primary cilia function as a sensory organelle in many eukaryotic cells. Recently several proteins have been seen to co-exist in primary cilia and in immune synapses of T cells, although how a rod-shaped cilium is related to a plate-shaped immune synapse remains elusive. Here we show that T-lymphocytes, which lack primary cilia, express inositol polyphosphate-5-phosphatase E (INPP5E), a ciliary-specific protein responsible for regulating phosphoinositide localization. We find that INPP5E colocalizes with the microtubule organizing center (MTOC) and the distal appendages in Jurkat T-cells. In addition, INPP5E accumulates at the immune synapse during antigen-specific conjugation. Knockdown of INPP5E results in abolished T-cell receptor (TCR) /CD3 recruitment at the immune synapse. Moreover, proximal TCR signaling, including CD3ζ and ZAP70 phosphorylation, is disrupted in INPP5E-deficient cells. The defects in INPP5E-deficient cells are contributed by phospholipid distributions at the supramolecular activation cluster (SMAC), which is modulated by the catalytic domain of INPP5E. Together, these results suggest a repurposed ciliary machinery at the immune synapse for the recruitment of the TCR/CD3 complex and regulated by controlling phospholipid distributions through INPP5E.

P1457/B589

Rafting with Rush: Membrane Rafts Mediate Protein Trafficking through the Biosynthetic Pathway.

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The organelles of eukaryotic cells maintain distinct protein and lipid compositions required for their distinct functions. However, the mechanisms by which components are sorted to their specific locations remain largely mysterious. In particular, how lipids and membrane proteins are coordinately delivered to various membrane-bound organelles is poorly understood. One proposed explanation is that the sorting of membrane components is mediated by membrane microdomains known as lipid rafts. Such

domains are small, dynamic clusters of preferentially interacting lipids and protein components. Recently, our lab has defined the structural determinants for protein partitioning to raft domains, identifying three independent aspects of protein transmembrane domains (TMDs) as key for raft affinity: TMD length, TMD surface area, and post-translational palmitoylation. Remarkably, all three were also correlated with protein subcellular localization, strongly suggesting a mechanistic link between raft affinity and protein sorting. To dissect the mechanisms of raft-mediated protein sorting, we have adapted the RUSH (Retention Using Selective Hooks) system to quantify the temporal dynamics of trafficking of transmembrane proteins through the secretory pathway and to the PM. We find that lipid raft affinity is necessary and sufficient for steady-state PM localization of a subset of transmembrane proteins. The TMDs of these proteins, encoding their raft affinity, were fully sufficient for PM sorting.However, raft affinity was not sufficient for rapid exit from the endoplasmic reticulum (ER), which required specific cytosolic sorting motifs. Importantly, we find that Golgi exit rates are highly raft-dependent, with raft preferring proteins exiting ~3-fold faster than mutants with perturbed raft affinity. We rationalize these observations with a mechanistic, predictive model of trafficking through the secretory pathway, which includes the partitioning of the Golgi into coexisting membrane domains. We identify an isoform of the small GTPase Rab6 as a central regulator for the Golgi-PM trafficking of raft proteins. These observations highlight a central role for lipid rafts in sorting in the secretory pathway and establish the core machinery for raft-mediated cellular trafficking.

P1458/B590

The Source of Intracellular Cholesterol Affects the Localization of Amyloid Precursor Protein and BACE in Cultured Cells.

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Ca^{2+}-independent phospholipase A_2 inhibitors (PLAIs) induce the accumulation of cholesterol in the Endocytic Recycling Compartment (ERC) but only when sufficient amounts of LDL are available to cells. Certain proteins that associate with cholesterol also accumulate in the ERC under these conditions, notably Amyloid Precursor Protein (APP). Immunoblotting revealed that the processing of APP and association of APP-derived fragments with detergent-insoluble complexes was altered by PLAI treatment. Whether the pathogenic form of A-beta was produced could not be determined, but cholesterol has been associated with altered processing of APP that produces the pathogenic form of the protein. The production of this version of A-beta requires the interaction of APP with BACE protease. Since PLAIs lead to the colocalization of cholesterol and APP, we investigated the colocalization of APP and BACE when cells were grown in different levels of LDL and treated with the PLAI, ONO-RS-082, or simvastatin. SH-SY5Y, HeLa, and NPC1 cells were grown in low, moderate, or high levels of LDL, then treated with ONO and simvastatin alone or in combination. Immunofluorescence confocal microscopy revealed colocalization of APP and BACE in a small fraction of juxtanuclear puncta only in untreated cells grown in low-LDL media. No colocalization was observed in cells grown in higher levels of LDL or treated

with ONO or simvastatin. This suggests that newly-synthesized cholesterol may affect the processing of APP, and how cells obtain cholesterol may influence cellular processes. Since PLA2 enzymes are associated with different compartments involved in membrane trafficking, these proteins may also play a role in the trafficking of newly-synthesized cholesterol and affect the trafficking or processing of APP.

P1459/B591

Exploring Cholesterol-dependent Membrane Interaction of a Beta-barrel Pore-forming Toxin *Vibrio Cholerae*Cytolysin and Its Association with Membrane Lipid Rafts.

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Vibrio cholerae cytolysin (VCC), an archetypical β-barrel pore-forming toxin, acts as a major virulence factor for the pathogenic strains of *Vibrio cholerae* that lack Cholera Toxin. VCC generates heptameric transmembrane pores in the eukaryotic cellular membranes. VCC has been shown to exhibit functional pore formation only in cholesterol-containing membrane bilayer. However, mechanistic details of how cholesterol governs functional activity of VCC have not been deciphered. The collective result from our study defines the critical requirement of membrane cholesterol in regulating the pore-forming activity of VCC. Our study provides detailed insights of how cholesterol in target membrane regulates the distinct steps of pore formation in both liposomes and eukaryotic biomembranes. Our findings for the first time demonstrated the marked tendency of VCC to partition into cholesterol-rich, membrane micro-domains or lipid rafts in human erythrocytes. Surprisingly, the variants of VCC having mutations that arrest the toxin at different stages of pore-formation mechanism, and even the mutant that could not interact with cholesterol tend to associate with lipid rafts of human erythrocytes with similar efficacy, suggesting that the sequestering of VCC to lipid rafts is possibly defined by the physicochemical environment of the membrane lipid rafts, or there could also be a possibility for presence of a yet unidentified specific receptor(s) or receptor-like entity in these lipid rafts regions that might drive the translocation of VCC to these regions for execution of pore formation.

P1460/B592

Multiple Sterol Routes to Lysosome/vacuole for Lipid Domain Formation.

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The composition of cellular membranes dramatically changes in response to stress. However, the mechanisms by which cells sense and regulate the composition and distribution of lipids in membranes remain poorly understood. During various stresses, such as glucose starvation, heat stress and weak acid stress, we have previously found that large, stable, sterol-enriched domains form in the membrane of the vacuole (lysosome-like organelle) in yeast. This is the first demonstration that sterol-enriched raft-like liquid ordered domains similar to those observed in artificial membranes form in living cells and have been linked to autophagy and Tor pathway. We found that these sterol-enriched domains are also enriched in phosphatidyl-inositol-3-phosphate (PI3P) and Diacylglycerol (DAG). The formation of these domains is tightly regulated and all three lipids are necessary for the formation of these lipid domains. In our current work, we reveal multiple additive routes for sterol to reach the vacuole membrane during glucose starvation: vesicular fusion (endocytosis), membrane degradation (autophagy, multivesicular

body, NPC proteins) and non-vesicular trafficking (ER/ mobilization from lipid droplets, lipid transfer proteins Ltc1/2p). In addition, we observed that similar lipid domains form in entotic structure found in human cancer cell lines and in lysosomes of Pompe disease mice model myotubes. Like the yeast vacuole, formation of these lipid domains depends on the presence of cholesterol and phosphatidylinositol phosphate. All together, these data show that the sterol-enriched domains are conserved from yeast to human and probably play an important role in stress response.

P1461/B593

Induced Hyperleptinemia in Caveolin-1 Null Mice Dramatically Decreased the Outer Plasma Membrane Amount of White Adipocytes.

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Caveolae are omega-shaped invaginations of plasma membrane rich in cholesterol and sphingolipids. Caveolin-1 is an important structural component of caveolae that are most abundant in adipocytes and other mammalian cell types. Adenovirus-induced hyperleptinemia rapidly depletes body fat in normal rats without increasing free fatty acids and ketogenesis, implying the fat is being oxidized in adipocytes. We induced hyperleptinemia in wild type (Wt) or Caveolin-1 (CAV-$1^{-/-}$) null mice with recombinant adenovirus containing the leptin cDNA followed by light and electron microscopic analysis. We found that leptinized adipocytes in both groups are shrunken, fat-depleted, crowded with mitochondria, and encased in a thick connective tissue matrix. Leptinized adipocytes of wild type revealed irregularly shaped shrunken cells with extreme folding of cell plasma membrane and also accumulated a large amount of caveolae just under the cell surface. Leptinized adipocytes of CAV-$1^{-/-}$ have no detectable caveolae which was observed previously. But CAV-$1^{-/-}$ adipocytes exhibited dramatically decreased cell surface plasma membranes, a lack of membrane folding and very few microvilli. The proliferation of filamentous mitochondria containing numerous cristae embedded in a highly electron-dense matrix were only seen in CAV-$1^{-/-}$ adipocytes. Our ultrastructural findings support the hypothesis that caveolae with Caveolin are integral to organizing and protecting the cell surface plasma membrane to response the mechanical changes.

P1462/B594

Diurnal Phosphatidylserine Exposure during Retinal Photoreceptor Outer Segment Renewal.

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Mammalian photoreceptor outer segment renewal is a synchronized circadian and light driven process that involves collaboration of photoreceptor neurons and neighboring retinal pigment epithelial (RPE) cells and that is essential for life-long visual function. Diurnal exposure of the anionic membrane lipid phosphatidylserine (PS) specifically at distal tips of rod photoreceptor outer segments triggers their shedding and clearance phagocytosis by RPE cells. RPE-photoreceptor communication is critical for synchronized outer segment renewal with outer segment PS recruiting bridge proteins such as MFG-E8 and Protein-S to efficiently ligate RPE receptors (αvβ5 integrin and Mer tyrosine kinase) triggering POS engulfment. This study aims to elucidate the molecular mechanisms promoting PS exposure by photoreceptors. Experiments involve characterization of wild-type and knockout mouse models by PS live imaging and immunofluorescence microscopy and immunoblotting of retinal and RPE marker

proteins. We show that the peak of PS exposure by photoreceptor outer segments in *vivo* at daily light onset requires the soluble carbohydrate-binding protein galectin-1. We also find that RPE cells express galectin-1 and that galectin-1 levels in the interphotoreceptor matrix are elevated at light onset, coinciding with maximal PS exposure by outer segments. Moreover, purified galectin-1 added exogenously is sufficient to promote outer segment PS exposure. However, the capacity of photoreceptor neurons to externalize PS at their tip is variable depending on time of day and is abnormal in retina with defective RPE phagocytic machinery. Altogether, our results identify an essential role for galectin-1 in synchronized photoreceptor outer segment renewal.

P1463/B595

Plasma Membrane Compartmentalization during Leader Bleb-based Migration in Melanoma Cells Under Confinement.

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Metastatic cancer cells migrating in a confined tissue microenvironment can switch between different modes of migration dependent on the degree of confinement and availability of adhesive ligands. Cells with high contractility are prone to blebbing and when confined in the absence of adhesive ligands, exhibit “leader bleb-based” migration. This mode of migration is characterized by a highly polarized morphology with a long, stable leader bleb (LB) leading the direction of migration, that is separated from the trailing cell body (CB) by a narrow contractile neck. Metastatic cells carrying the BRAFV600E mutation exhibit polarized Erk signaling within the LB that promotes actin retrograde flow to drive fast, directional cell LB-based motility. However, the mechanisms mediating the establishment and maintenance of such extreme cell polarization during LB-based migration are not known. We seek to test the hypothesis that polarization of signaling during LB-based migration is mediated by compartmentalization of lipid and protein organization on the plasma membrane (PM) between LB and CB. To test this, we expressed fluorescent membrane proteins and lipid probes in human metastatic A375 melanoma cells under 3µm confinement with a polydimethylsiloxane (PDMS) or an agarose pad. We performed FRAP and time-lapse confocal microscopy to analyze mobility and spatial distribution within LB and CB, as well as cell motility parameters. To address the role of protein topology relative to the membrane in the LB and CB, we analyzed fluorescent fusions of single-pass transmembrane proteins (GFP-GT46 for non-raft, LAT-GFP for raft), those associated with the outer leaflet (GFP-GPI). Localization and FRAP photobleaching experiments revealed that transmembrane proteins showed restricted diffusion between LB and CB while proteins on the outer leaflet were free to diffuse. An alysis of an inner leaflet protein (HRas-GFP) as well as a transmembrane proteins associated with cytoskeleton (CD44-GFP) is underway. To address the role of lipid organization in the LB and CB, we analyzed the distribution of phosphoinositides using biosensors (PLCδ PH-GFP or tubby domain-GFP for $PI[4,5]P_2$, Akt PH-GFP for $PI[3,4,5]P_3$). This showed that PLCδ PH-GFP labeled the PM evenly, while tubby domain-GFP and Akt-GFP was excluded from the blebs. An alysis of lipid order using laurdan and Di-4-AnePPDHG dyes will be performed in the future. However, our results together reveal a striking degree of PM compartmentalization between LB and CB in melanoma cells migrating under confinement, though the mechanisms behind this still remains elusive. We are currently deciphering the mechanism by which such a compartmentalization is attained and how membrane organization regulates bleb dynamics and migration.

P1464/B596

Development of Ph-sensitive Fluorescent Probes for Imaging of Cell Membranes in Live Cells.

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Biomembranes are essential to life and the understanding of lipid membranes is of fundamental interest for cell biology, biochemistry and drug design. The composition of lipid membranes depends on the membrane type, localization and cell type. Certain neurodegenerative diseases and cancers relate to disfunction of biomembranes and their components. Lipid membranes of cancer cells present substantial alterations in their composition, structural organization, and functional properties. Disturbance of processes related to lipid trafficking is considered a basis for some neurodegenerative diseases such as Alzheimer's. Reported here is the synthesis, characterization and application of novel pH sensitive probes for lipid membrane imaging. As a fluorescent probe the 1,8-naphthalimide scaffold was chosen. The 4-amino(substituted)-1,8-naphthalimide derivatives have very interesting electronic properties and ability to respond to internal charge transfer (ICT) excited state. Moreover, the ICT is also solvent-dependent process, giving rise to interesting solvatochromic properties of naphthalimide derivatives. Reported in this study are the probes containing substituted naphthalimides with aliphatic chains, probes containing cholesterol and amino-acid residues to be used in peptide synthesis for transmembrane peptides. The designed probes (λex ~400 nm, λem ~520 nm) are not only membrane specific but also increase fluorescence (several times) in slightly acidic environment (pH~6.5). Performed molecular dynamics simulations of probes in membrane models show how probes incorporate into lipid bilayers and interact with it. Two of the tested probes found their application for the imaging of lipid membranes of live (breast) cancer cells with an excellent signal-to-background ratio of ~1200-1500. Additional probe containing cholesterol moiety was designed to study cholesterol rich membranes as those present in neurons and synaptic vesicles. This probe is capable of interacting with lipids and transmembrane proteins through its cholesterol fragment and "head" piperazine moiety. An other naphthalimide moiety was also coupled to a lysine residue, resulting in fluorescent amino acid derivative that serves as a building block for synthesis and labeling of transmembrane peptides. The model transmembrane peptide when tested in micellar solution showed an ideal alpha-helix formation and green fluorescence emission. The physicochemical and spectral properties of all fluorescent probes is discussed and illustrated with a potential application towards imaging of lipid membranes of live cancer cells and neurons.

P1465/B597

Rafting with Rush: Membrane Rafts Mediate Protein Trafficking through the Biosynthetic Pathway.

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The organelles of eukaryotic cells maintain distinct protein and lipid compositions required for their distinct functions. However, the mechanisms by which components are sorted to their specific locations remain largely mysterious. In particular, how lipids and membrane proteins are coordinately delivered to various membrane-bound organelles is poorly understood. One proposed explanation is that the sorting of membrane components is mediated by membrane microdomains known as lipid rafts. Such

domains are small, dynamic clusters of preferentially interacting lipids and protein components. Recently, our lab has defined the structural determinants for protein partitioning to raft domains, identifying three independent aspects of protein transmembrane domains (TMDs) as key for raft affinity: TMD length, TMD surface area, and post-translational palmitoylation. Remarkably, all three were also correlated with protein subcellular localization, strongly suggesting a mechanistic link between raft affinity and protein sorting. To dissect the mechanisms of raft-mediated protein sorting, we have adapted the RUSH (Retention Using Selective Hooks) system to quantify the temporal dynamics of trafficking of transmembrane proteins through the secretory pathway and to the PM. We find that lipid raft affinity is necessary and sufficient for steady-state PM localization of a subset of transmembrane proteins. The TMDs of these proteins, encoding their raft affinity, were fully sufficient for PM sorting.However, raft affinity was not sufficient for rapid exit from the endoplasmic reticulum (ER), which required specific cytosolic sorting motifs. Importantly, we find that Golgi exit rates are highly raft-dependent, with raft preferring proteins exiting ~3-fold faster than mutants with perturbed raft affinity. We rationalize these observations with a mechanistic, predictive model of trafficking through the secretory pathway, which includes the partitioning of the Golgi into coexisting membrane domains. We identify an isoform of the small GTPase Rab6 as a central regulator for the Golgi-PM trafficking of raft proteins. These observations highlight a central role for lipid rafts in sorting in the secretory pathway and establish the core machinery for raft-mediated cellular trafficking.

P1466/B598

Interaction of Msc1 with Yy1 Is New Target of Sarcopenia.

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In modern society, obesity is the leading cause of many complications such as diabetes and hyperlipemia. A typical phenomenon is that as the amount of fat increases, muscle mass gradually decreases, damaging many cells. YY1(YinYang1) is a transcription factor belonging to the GLI-Kruppel of zinc finger proteins. YY1 has been extensively studied for muscle regeneration. YY1 is an important factor in muscle differentiation. The muscle-specific deletions of YY1 lead to severe deformities of muscle formation, resulting in neonatal death. It was also reported that the deficiency of YY1 induced by satellite cells (SC) almost completely inhibited muscle repair induced by acute injury and worsened the phenotype of malnutrition caused by chronic injury. MSC1 is Methyllysine-binding protein, a component of the MOF histone acetyltransferase protein complex. MSC1 phosphorylation after DNA damage contributes to the down-regulation of p53 / TP53 We found that insulin resistance and muscle regeneration were reduced when the MSC1 TG mice feed with high fat diet (HFD). Moreover cardiotoxin-induced muscle regeneration in MSC1 Tg mice was also decreased. In addition, IP assay indicated that MSC1 interacts with YY1. The relationship between MSC1 and YY1 were confirmed through H & E and behavior test. This results implicated that MSC1 might be a targeting mediator for the treatment of sarcopenia.

Post-Translational Modifications in Signaling

P1467/B600

Casein Kinase I Protein Hrr25 Mediates Cell Wall Integrity by Phosphorylating Pin4 in Budding Yeast.

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Casein kinase I protein Hrr25 plays important roles in a number of cellular processes including autophagy, vesicular trafficking, ribosome biogenesis, transcriptional regulation, and DNA damage response in the budding yeast. In a bioinformatics analysis of genes that genetically or physically interact with Hrr25, we found that Pin4 was a potential target of Hrr25. Pin4 is a multiply phosphorylated protein. We found that its phosphorylation is dependent on Hrr25. Pin4 has been reported to be involved in both DNA damage response and the cell wall integrity pathway. By performing a co-immunoprecipitation analysis and a yeast two-hybrid assay, we found that Pin4 physically interacts with Hrr25. *pin4* mutations form synthetic slow growth phenotypes with mutations in genes encoding Bck1 and Slt2, two of the protein kinases in the MAK kinase cascade that regulates cell wall integrity in the budding yeast. We found that *hrr25* mutations result in similar phenotypes to those of *pin4* mutations. Hrr25 possesses an N-terminal kinase domain, a middle region, and a C-terminal proline/glutamine rich domain. The function of the C-terminal P/Q rich domain of Hrr25 has been elusive. We found that the C-terminal region of Hrr25 is required for its interaction with Pin4 and that an *hrr25* mutant allele with the C-terminal region truncated forms synthetic growth defects with mutations in *BCK1* and *SLT2*. Our results suggest that Hrr25 positively regulates cell wall integrity by phosphorylating and activating Pin4.

P1468/B601

Phosphorylation of Nur77 by Tak1-p38 Mapk Pathway Regulates the Inflammatory Response.

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Nur77 is a member of the NR4A subfamily of nuclear receptors and has been shown to regulate various biological processes such as inflammation and apoptosis. The aim of current study is to determine whether Nur77 phosphorylation regulates their anti-inflammatory effects. Overexpression of Nur77 inhibited IL-6 production by TNFα, whereas knockdown of Nur77 amplified IL-6 production. Treatment of TNFα increased phosphorylation of two serine residues of Nur77 protein, S152 and S351 in HeLa cells. Next, we examined the signal transduction pathway regulating phosphorylation of Nur77. Inhibition of p38 MAPK decreased both S152 and S351 phosphorylation of Nur77 as well as IL-6 production. Suppression of TAK1 activity by siRNA or inhibitor decreased phosphorylation of Nur77 as well as p38 MAPK. In addition, TAK1 knockdown or inhibitor suppressed TNFα-induced IL-6 production. These results suggest that TAK1-p38 MAPK pathway regulates phosphorylation of Nur77. We investigated that S152 phosphorylation of Nur77 regulates IL-6 production. In the IL-6 production of TNFα stimulation, the S152A mutant suppressed more strongly than the WT Nur77, while the S351A mutant did not affect. These results suggest that although TNFα stimulates phosphorylation of S152 and S351 of Nur77 through TAK1-p38 MAPK pathway, only phosphorylation of S152 reduces the stabilization of this protein and eventually interferes the anti-inflammatory effect.

P1469/B602

Mating-induced Stabilization of Kar4p by Down-regulation of the E3-ubiquitin Ligase Ubr1p.

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Signal transduction pathways respond to changes in a wide variety of external conditions to control cellular processes, such as proliferation, differentiation and development. Given that there are a limited number of signaling proteins, cells have evolved an elegant mechanism to maintain specificity between different developmental programs that share identical or similar regulators. In addition to phosphorylation to regulate the activities of signaling proteins, emerging evidence has shown a key role of ubiquitination and turnover in modulating the strength and duration of signaling pathways. In budding yeast, Kar4 is a multi-functional protein that has an essential role in various cellular processes with distinct functions. The protein is expressed in two forms. The long form is constitutive and responsible for meiosis and sporulation; the short form, initiated from an internal AUG, is highly induced by pheromone and Ste12 during mating to regulate karyogamy. Kar4p-short is stable during mating, when it complexes with Ste12 and transcriptionally induces the minus end-directed kinesin motor proteins Kar3/Cik1. Kar4-short is then rapidly degraded as cells return to mitosis. Here, we demonstrate that the pheromone signaling pathway regulates the Ubr1 E3-ubiquitin ligase. In mitosis, *ubr1Δ* mutation stabilized Kar4-short to the level of pheromone-treated cells. A *ubr1* RING-domain mutation also stabilized Kar4-short. Regulated turnover is not limited to Kar4; other Ubr1 substrates (C-terminal fragment of Scc1 and misfolded Ste6*) also showed an increase in turnover rate in mating cells, indicating that pheromone signaling down-regulates the Ubr1 pathway. An allele of Kar4 that does not bind to Ste12 failed to stabilize Kar4 during mating. A subsequent screen of Kar4-dependent genes identified Srl4 as being required for stabilization during mating; in pheromone-treated *srl4*Δ, the turnover rate of Ubr1 substrates was similar to the rate in mitotic cells, and mitotic cells overexpressing Srl4 showed reduced turnover. Consistent with this, sr*l4Δ* lowered the level of Kar4 and Kar3, which led to mating defects. Ubr1 is an N-end rule ligase that normally requires the canonical E2-conjugating enzymes Ubc2/Rad6. Interestingly mutation of Ubc2 and Rad6 did not affect Kar4 stability. Instead, deletion of a different E2, Ubc6, caused mitotic stabilization of Kar4 similar to *ubr1*Δ, suggesting that Kar4 is degraded by an alternate pathway. LC-MS showed that both Kar4 and Ubr1 are complexed with Srl4 in pheromone treated cells. However, no E2-ligases, including Ubc6, were detected. We are now testing that Kar4-induced Srl4 can block access of the E2-conjugating enzyme to Ubr1, thereby causing down-regulation of Ubr1 and stabilization of Kar4 during mating.

P1470/B603

The Functional Role of Calcineurin Regulating Factor 1 in β-amyloid Generation in Alzheimer's Disease.

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Alzheimer's disease (AD) is the most common neurodegenerative disease. The excessive generation of Aβ plays a major role in the pathogenesis of AD. The dysregulation of amyloid precursor protein cleaving enzymes, β-secretase and γ-secretase, is an important cause of AD. RCAN1.1L has been demonstrated to be up regulated in AD brains, which promotes neuronal apoptosis mediated by caspase 3 activation. In this work, we demonstrated the expression of BACE1, a dominated β-secretase leading to Aβ generation, was modulated by RCAN1.1L. Moreover, we also found that the expression of factor F was also regulated by RCAN1.1L in a calcineurin dependent pathway, which was involved in Aβ generation.

Conserved recognition motif of factor F was widespread in several amyloid precursor protein cleaving enzymes, including BACE1, indicating its potential role in Aβ accumulation via mediating degradation of BACE1. Our study suggests that RCAN1.1L might be implicated in Aβ generation by regulating factor F-mediated BACE1 degradation. This work is beneficial to further elucidate functional role of RCAN1.1L in pathogenesis of AD and may provide new potential targets for its treatment.

P1471/B604

Phosphorylation-dependent Regulation of the GLI Family of C2H2 Zinc Finger Transcription Factors.

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C2H2 zinc finger transcription factors (ZNTFs) are the largest family of transcription factors found within eukaryotes. How post-translational modification of ZNTFs regulates activity is still poorly understood. Our previous studies indicated that phosphorylation of the first and third zinc finger of the Hedgehog (Hh) pathway ZNTF GLI1 positively regulates DNA binding and transcriptional activity. To explore phospho-regulation of this ZNTF, we undertook a brute-force mutagenesis of every serine and threonine residue and found three conserved regions of regulation: 1) a permissive regulatory region, 2) a DNA binding region, and 3) a cell-cycle regulatory region. Specific phospho-mimetic mutations in the regulatory region increases DNA binding or transcriptional activity of GLI1, whereas phospho-mimetic mutations in the DNA binding or cell-cycle region abolishes DNA binding. We find these regions are conserved in other GLI family members and ZNTFs with similar zinc finger structure, suggesting a broadly conserved mechanism to post-translationally control ZNTF activity.

P1472/B605

Prmt1-mediated Tsc2 Methylation Controls Tsc2 Stability and Mtorc1 Activity.

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[Objective] Insulin signals regulate cell growth by activating mammalian target of rapamycin complex 1 (mTORC1). Tuberous sclerosis complex 2 (TSC2), a tumor suppressor protein, is the intermediator of the insulin pathway and negatively regulates mTORC1 activity. The post-translational modifications of TSC2, such as TSC2 phosphorylation, inactivate TSC2 during the insulin stimulus. Protein methylation is a well-known protein modification wherein arginine methyltransferase 1 (PRMT1) methylates arginine residues on the Gly- and Arg-rich (GAR) motif. However, although TSC2 contains several GAR motifs, TSC2 methylation is poorly understood. This study aimed to characterize TSC2 methylation and its function. **[Methods]** Initially, the potential PRMT1-mediated TSC2 methylation was investigated by in vivo methylation assay. Next, we confirmed whether TSC2 stability is controlled by its PRMT1-mediated methylation. We further examined whether the TSC2 decrease through arginine methylation affects mTORC1 activity. Finally, we analyzed whether the decrease in TSC2 is controlled by methionine metabolism via the supplementation of S'-adenosyl methionine (SAM). Moreover, we investigated TSC2 localization by immunofluorescence. **[Results and Discussion]** to the best of our knowledge, this is the first study to report that TSC2 is directly methylated by PRMT1. In vivo methylation assay results indicated that PRMT1 inhibitor treatment decreases TSC2 methylation. Additionally, treatment with the PRMT1 inhibitor treatment and shPRMT1 KD cells decreased endogenous TSC2, which, in turn, promoted mTORC1 activity, as measured by S6K phosphorylation. These findings implied that PRMT1-mediated TSC2 methylation regulates cell growth through mTORC1 inactivation. The supplementation of SAM prevented a decrease in TSC2 upon methionine deprivation. In addition, TSC2 localization on

lysosomes significantly decreased upon methionine deprivation. **[Conclusion]** We identified a novel protein modification for TSC2 methylation. Inhibition of TSC2 methylation leads to TSC2 degradation. Moreover, methionine regulates TSC2 stability through TSC2 methylation.

P1473/B606

Optogenetic Control of Subcellular O-GlcNAc Signaling.

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O-GlcNAcylation is a post-translational modification that regulates fundamental cellular processes in metazoans. A single pair of enzymes - O-GlcNAc transferase (OGT) and O-GlcNacase (OGA) - mediates dynamic cycling of O-GlcNAcylation on a wide variety of nucleocytoplasmic and mitochondrial proteins in a nutrient- and stress-responsive fashion. A significant hurdle towards understanding O-GlcNAcylation in cells arises from the lack of tools to perturb O-GlcNAcylation levels with spatiotemporal precision. To overcome these challenges and identify the role of O-GlcNAcylation in the subcellular context, we develop a set of optogenetic tools based on light-induced protein oligomerization to control the O-GlcNAcylation levels at the cellular and organelle levels. Here, we demonstrate that light-induced oligomerization of CRY2-OGT rapidly raised the overall O-GlcNAcylation levels in HEK 293 cells within 5 minutes. We then utilize the heterodimerization property of CRY2 and CIBN to recruit OGT to various organelles, including mitochondria. The optogenetic recruitment of OGT elevates mitochondrial O-GlcNAcylation levels and increases fusion events. This is associated with increased phosphorylation of dynamin-related protein 1 (DRP1) at Ser 637 and reduced phosphorylation at Ser 616. These results demonstrate the direct role of O-GlcNAc signaling in regulating mitochondrial dynamics and function. Our work describes a novel tool to study O-GlcNAcylation with high spatiotemporal specificity and provides insights into the role of O-GlcNAcylation at the subcellular level.

P1474/B607

Acinus Supports Atg1-mediated Phosphorylation of Yorkie to Restrict Cell Growth.

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Acinus has emerged as a signaling node that integrates multiple cellular stress signals and manages stress by regulating basal levels of autophagy in a TOR-independent pathway (1,2,3). Here, we identified Acinus-supported Atg1/ULK1-mediated phosphorylation of Yorkie as an additional inhibitory input restricting growth independently of the Hippo/Warts pathway. Two serine residues in Yorkie, S74 and S97, are Atg1/ULK1 consensus target sites phosphorylated by ULK1 in vitro. In vivo, Atg1 gain-of-function caused elevated Yorkie phosphorylation and reduced expression of Yorkie target genes. Conversely, knocking down Atg1, or its activator Acinus, reduced Yorkie phosphorylation and raised expression of Yorkie target genes to increase tissue size. Yorkie phosphorylated by ATG1 can still translocate to the nucleus, but was unable to bind the transcription factor Scalloped and failed to promote transcription of pro-proliferation genes, thereby restricting cell growth. Unlike Atg1's role in autophagy, Atg1-mediated phosphorylation of Yorkie does not require Atg13. Atg1 is activated by starvation or other cellular stressors and therefore can impose temporary stress-induced constraints on the growth-promoting gene network under control of Hippo/Yorkie signaling.

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3. Nandi et al (2017) eLife 6:e30760

P1475/B608

An In-cell Sensor Reveals the Spatiotemporal Dynamics of Phosphorylated Gbeta in Yeast Cells Decoding Pheromone Gradients.

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Directed cell growth in response to a chemical gradient (chemotropism) is necessary for cellular processes like axonal pathfinding, angiogenesis and fungal life cycles. Yeast mating is a well-studied chemotropic model system. Yeast cells interpret shallow pheromone gradients from cells of opposite mating type, polarize their growth toward the pheromone source, and fuse at the chemotropic growth site. When cells are unable to sense a pheromone gradient, they can fuse at the default polarity site. To accurately decode pheromone gradients, mating cells first assembly signaling, polarity, and trafficking proteins (the gradient tracking machine, GTM) at the default polarity site. The GTM then redistributes (tracks) toward the pheromone source until it reaches the chemotropic growth site. Phosphorylation of the G protein βετα subunit (Gβ) is important for this process. Phosphorylated Gβ ($G\beta^P$) inhibits receptor phosphorylation up-gradient promoting differential phosphorylation of the receptor, which is critical for gradient sensing. We postulate that gradient tracking depends on differential phosphorylation of the receptor,which in turn, depends on localized phosphorylation of Gβ. To test this prediction, we developed a biosensor that specifically recognizes phosphorylated forms of Gβ. Here, we describe the isolation and characterization of the $G\beta^P$ biosensor. We show that in mating cells expressing both RFP-Gβ and the $G\beta^P$ biosensor, the spatiotemporal behavior of gradient-tracking $G\beta^P$ relative to that of total gradient-tracking Gβ is consistent with our proposed role for Gβ phosphorylation in gradient tracking.Notably, we found that $G\beta^P$ anticipates the GTM transition from tracking to stabilization, suggesting that it positions the chemotropic growth site.

P1476/B609

The Interplay between O-glcnac and Phosphorylation on Tyrosine Hydroxylase Activity and Cathecolamine Synthesis in Pc12 Cells.

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About 2% to 5% of all glucose that enters the cell are directed to the Hexosamine biosynthetic pathway, which has glutamine fructose-6-phosphate aminotransferase (GFAT) as the rate-limiting step enzyme. This pathway has UDP-GlcNAc as a final product, which is used as substrate for intracellular O-GlcNAc; a post-translational modification (PTM) resulting from the covalent attachment of a N-acetylglucosamine (GlcNAc) to the hydroxyl groups on serine and threonine residues in proteins. This reaction is catalyzed by O-GlcNAc transferase (OGT), and the removal reaction of this monosaccharide is made by O-GlcNAcase (OGA). The balance of the activity of such enzymes and UDP-GlcNAc availability will regulate the levels of O-GlcNAcylated proteins, with an O-GlcNAcylation, similarly to phosphorylation, highly inducible, dynamic and active in many cellular processes. Tyrosine hydroxylase (TH) is the rate-limiting step enzyme in catecholamine synthesis, responsible for hydroxylate L-tyrosine at meta-position to obtain L-DOPA, the precursor of dopamine, which has a physiological role as a neurotransmitter. There

are several supports on the literature that phosphorylation on serine 40 of TH increases its activity on a dynamical way, however, the mechanism of how O-GlcNAc plays a role on this modulation remains unknown. We showed in PC12 cells treated with the pharmacological inhibitor of OGA Thiamet G (TMG); and nerve growth factor (NGF), a compound that induces these cells in a process of neuritogenesis, that O-GlcNAcylation acts on the control of the phosphorylation levels of serine 40 in TH, where stimulation by 28% on the increase in phosphorylation at serine 40 decreases the levels of O-GlcNAc in 26% compared to control; while the increase of intracellular O-GlcNAc in 19% reduces the phosphorylation at serine 40 by 16%. In addition, HPLC analysis shows that the increase of intracellular O-GlcNAc reduces the levels of L-DOPA and dopamine by 80%. Finally, TH's immunoprecipitation analysis reveals that it is O-GlcNacylated, and this PTM regulates its activity. These data suggest a cellular mechanism that integrates carbohydrate metabolism (by HBP) with the catecholamine biosynthetic pathway in a neuritogenesis process; where competition between O-GlcNAc and phosphate at serine 40 tyrosine hydroxylase site modulates its activity, controlling the synthesis of dopamine levels in PC12 cells.

P1477/B610

Proteomic an alysis Identifies the E3 Ubiquitin Ligase Pdzrn3 as a Regulatory Target of Wnt5a-Ror Signaling.

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WNT5A-ROR signaling is a conserved pathway that regulates morphogenetic processes during vertebral embryonic development, but the downstream signaling events remain poorly understood. Using a large-scale proteomic screen, we identified the E3 ubiquitin ligase PDZRN3 as a new regulatory target of WNT5A-ROR signaling that is degraded upon pathway activation in a β-catenin independent, ubiquitin proteasome system dependent manner. To track this regulation, we developed a fluorescence-based reporter to monitor PDZRN3 abundance in live cells. By coupling this reporter with pharmacological perturbation and protein overexpression experiments, we delineated a signaling cascade involving Frizzled receptors, Dishevelled scaffolding proteins, Glycogen synthase kinase and Casein kinase 1 that leads to PDZRN3 degradation. Genetic epistasis analysis suggests that PDZRN3 resides in a branch of WNT5A-ROR signaling parallel to KIF26B, a previously identified regulatory target. Further, we discovered that PDZRN3 degradation requires WNT5A-dependent phosphorylation of its C-terminal LNX3H domain, and PDZRN3 homologs that contain a LNX3H domain are similarly regulated by WNT5A. Collectively, this work establishes a new WNT5A-ROR signaling cascade involving PDZRN3 phosphorylation and degradation.

P1478/B611

Akt Activation by Ptdins(3,4)p_2-dependent Mtorc2 Recruitment into Early Endosomes.

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The serine/threonine kinase AKT is a major effector during cell signal transduction in response to extracellular stimuli. AKT regulates cell physiological processes such as survival, growth and metabolism and is hyperactivated in metabolic diseases and cancer. AKT activation mechanisms have been extensively studied, but the mTORC2 mechanism that leads to AKT phosphorylation at Ser473 remains

elusive. Here, we investigated the upstream regulation of mTORC2 with confocal imaging and biochemical fractionation. We reveal that mTORC2 components are localized to the early endosome in cells activated with growth factor. The association of mTORC2 with early endosomes is responsible for local activation of AKT, which is critical for specific signal transduction through GSK-3β. Using inducible endosomal targeting lipid phosphatases, we found that endosomal PtdIns(3,4)P_2 provides a binding platform for mTORC2 to phosphorylate AKT at Ser473 on endosomes through mSIN, a PH domain-containing protein, and is dispensable for AKT phosphorylation at Thr308.

P1479/B612

Cell Cycle-dependent Regulation of Cyclic GMP-AMP Synthase (cGAS) Activity.

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In eukaryotic cells, DNA is sequestered in the nucleus and mitochondria. The presence of DNA in the cytoplasm triggers an immune response by activating the innate immune sensor cyclic GMP-AMP synthase (cGAS), which binds DNA from invading pathogens. cGAS localizes to self-DNA during mitosis and after mitotic errors that result in cytoplasmic DNA. The outcomes of cGAS localization in these different contexts are unique. Although nuclear envelope breakdown exposes chromosomes to cytosolic cGAS, mitotic cells do not produce cGAMP. Furthermore, cGAMP production in mitotic cell lysates cannot be stimulated by the addition of exogenous DNA in *vitro*. This indicates that regulatory mechanisms exist to downregulate cGAS-mediated cGAMP accumulation in mitosis. I will determine these regulatory mechanisms by identifying post-translational modifications and binding partners unique to cGAS in mitotic cells. In contrast, cGAS initiates an immune response following mitotic errors that result in aneuploid daughter cells. An euploidy contributes to tumorigenesis by providing a fitness advantage under specific circumstances that allows for the proliferation of cancerous cells. cGAS-driven recognition of aneuploid cells may allow for their removal. This hypothesis is being tested by monitoring aneuploidy levels and tumorigenesis in a cGAS knockout mouse model.

P1480/B613

Low Birth Weight Correlates with Elevated Circulating Interferon-gamma and Vaccine Unresponsiveness Phenotype in Pigs.

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Vaccine-induced immunity to infectious disease can vary significantly between outbred animals and humans. An imals which fail to develop a protective response to vaccination remain at risk for infection while also endangering the population by compromising herd immunity. While a part of the solution is in the development of more effective vaccines, the ability to anticipate responsiveness prior to vaccination could also improve herd health by enabling alternate strategies for disease management. In the current investigation, piglets (n=117) were immunized with a commercial vaccine (Respisure-ONE), and the

vaccine-induced antibody titer was quantified through ELISA. Subpopulations of animals (n=6) representing the high and low extremes of responses were investigated to identify biomarkers and molecular mechanisms of vaccine responsiveness. Kinome analysis performed on peripheral blood mononuclear cells collected immediately prior to vaccination identified 53 differential phosphorylation events ($p<0.05$) between the high and low responders. Pathway over-representation analysis indicated elevated pro-inflammatory cytokine signalling in the low responders prior to vaccination. Consistent with this finding, low responders exhibited higher ($p<0.01$) levels of plasma interferon-gamma compared to the high responders. In addition, low responder pigs had lower ($p<0.01$) birth weights compared to high responders. Within the high and low responders, but not the entire population of animals, there were correlations between birth weight and antibody titer ($r=0.84$, $p<0.005$), and birth weight and plasma IFNγ ($r=-0.67$, $p<0.05$). The results presented suggest vaccine responsiveness may be impacted by birth weight and pre-vaccination cytokine concentrations, collectively strengthening the evidence for determining a biomarker of vaccine responsiveness.

P1481/B614

N-terminal Cleavage of iκbα Ameliorates Er Stress in Cardiac Myocytes.

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Canonical ER (endoplasmic reticulum) stress sensor, PERK (protein kinase-like kinase), involves cardiac hypertrophy and heart failure. The importance of IκBα/NF-κB signaling pathway in cardiac hypertrophy has been well known. Here, we describe the emerging role of IκBα on ER stress in cardiac hypertrophy. Proteasome inhibitor, MG132, targeted protein degradation by the ubiquitin-proteasome system (UPS) leads cleavage of IκBα at N-terminus as shown in our previous study with HSP90 inhibitor, geldanamycin, treatment. The N-term cleavage of IκBα by geldanamycin was inhibited when H9c2 cardiac myoblasts were treated with a caspase-8 inhibitor, z-IETD-fmk. Silencing of caspase-8 in geldanamycin-treated cells effectively blocks the cleavage of IκBα. To examine the role of cleavage of IκBα in ER stress, the putative caspase-8 cleavage site of Asp31 was mutated to Ala. Cleavage at N-term of IκBα (WT) under geldanamycin treatment did not occur in IκBα (D31A) and the cleavage-deficient mutant enhances the expression of PERK. These results suggest N-terminal cleavage of IκBα ameliorate ER stress in cardiac cells, providing an emerging role of IκBα cleavage for cardiac hypertrophy and heart failure.

P1482/B615

Modeling Parietal Cell Physiology in Gastric Organoids Unraveled the Role of Alpk1-acapin Interaction.

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Digestion in the stomach depends on acidification of the lumen. Histamine-elicited acid secretion is triggered by activation of the PKA cascade, which ultimately results in the insertion of gastric H,K-ATPases into the apical plasma membranes of parietal cells. Our recent study established gastric organoid model to delineate the molecular signaling underlying parietal cell secretion (Liu et al., 2019. *J. Mol Cell Biol.* doi: 10.1093/jmcb/mjz051; Yao & Smolka. 2019. *Gastroenterology*. 156, 2158-2173.). Here we show that acapin, an ARF6-ACAP4 interacting protein (Song et al., 2018. *J. Mol Cell Biol.*10, 559-572), interacts with ALPK1 kinase and elevates ARF6 GTPase gradient at the apical membrane for H,K-ATPase recruitment. Acapin physically interacts with ALPK1 and is a cognate substrate of ALPK during parietal cell activation. The phosphorylation site of acapin by ALPK1 was mapped by mass spectrometric

analyses. Importantly, phosphorylation of acapin is essential for acid secretion in parietal cells because either suppression of acapin or overexpression of non-phosphorylatable acapin prevents the apical membrane reorganization and proton pump translocation elicited by histamine stimulation. Mechanistically, phosphorylation of acapin enables its interaction with ACAP4-ezrin complex for apical establishment. Taken together, these results define a novel molecular mechanism linking the MST4-ALPK1-acapin signaling cascade to apical establishment and polarized acid secretion in gastric parietal cells.

8

Rho-family GTPases

P1483/B616

Rho GTPase and DNA Repair: a New P53-dependent Road for IR-resistance of Glioblastomas?

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The Rho GTPases regulate several cellular processes related to tumor progression. The differential expression and activity of Rho GTPase family members dictates invasive strategies in glioblastoma (GBM), the most lethal cancer of the CNS, and are related to their malignancy. Despite aggressive therapies combining surgery and radio-chemotherapy, an effective treatment for these tumors remains obscure. We proposed that Rho GTPase pathway is modulating the genomic stability of GBM and their resistance to γ-radiation (IR) in a p53-dependent way. U87-MG (p53 wild-type) and T98G (p53 mutated) cells were subjected to Rho pathway inhibition by C3 toxin, knockdown of Rho pathway components mDia-1, Profilin-1 and MYPT1 or inhibition of ROCK by Y-27632 inhibitor. Comet assays showed that Rho inhibition by C3 and knockdown of mDia-1 and Profilin-1 led to increased DNA damage breaks and a delayed DNA repair after IR in U87-MG but not in T98G cells. The knockdown of MYPT1 reduced the amount of DNA breaks in both cells. The inhibition of ROCK sensitized the U87-MG while increased the resistance of T98G cells to IR. Immunofluorescence showed that foci formation of γH2AX and 53BP1 was compromised by Rho pathway inhibition and enhanced by its activation, with a pronounced effect in U87-MG cells. The impairment of Rho pathway increased the levels of phospho-Chk2 and decreased the phosphorylation of ATM, BRCA1 and NBS1 after IR in U87-MG cells only. Specific functional assays for the HR and NHEJ repair pathways showed that the impairment of Rho signaling decreased the DSBs repair by NHEJ only in U87-MG cells, while affected the HR repair in both cells. Conversely knockdown of MYPT1 increased the repair by HR in both cells while ROCK inhibition increased HR repair in T98G and had the opposite effect in U87-MG cells. These results indicate a striking regulatory relationship between Rho GTPase, DDR and HR/NHEJ repair pathways. Bioinformatics analysis in public databases indicated that high expression of RHOA and RHOC, as well as some of their downstream components, is closely related to aggressiveness of gliomas and a shortened patient survival. Comparative analysis of Rho activity in cells expressing wild-type or different p53-mutants showed that p53 mutated cells were more resistant to IR after Rho inhibition. An alysis of somatic mutations from databases showed that U87-MG cells has mutations in genes involved in DNA repair, actin remodeling dynamics and cell cycle regulation. Pearson correlation analysis showed that expression of this mutated genes and rho gene are positively correlated in GBM patient's samples. This work thereby shows the Rho pathway might be a fragile point in the resistance of gliomas against the usual therapies, being this effect very likely dependent on p53 transcriptional activities.

P1484/B617

Extraction of Active Rhogtpase by Rhogdi Regulates Rhogtpase Patterning.

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The subcellular localization and activity of the RhoGTPases are tightly regulated to ensure proper spatial control of processes such as cytokinesis, cell migration and cell wound repair. Classically, the RhoGTPases are characterized as membrane-associated molecular switches cycling between active, GTP-bound and inactive, GDP-bound states. The transitions between these two states are thought to be predominantly controlled by RhoGTPase regulators - GEFs and GAPs - that modulate interactions with the associated nucleotide. Yet at any given moment, 90-95% of RhoGTPases are not associated with membranes, but maintained in a soluble form by a third regulator named RhoGDI. RhoGDI is generally viewed as a passive shuttle for inactive RhoGTPases. Our current understanding of RhoGTPase:RhoGDI dynamics has been limited by two major experimental challenges: the direct visualization of the RhoGTPases in *vivo and* the reconstitution of the cycle in *vitro*. To study the regulation of RhoGTPases by RhoGDI, we developed methods to directly image vertebrate RhoGTPases both in *vivo* in the *Xenopus* oocyte wound repair model or on lipid bilayers *in vitro*. Using these tools in parallel, we identified pools of both active and inactive RhoGTPase associated with the membrane. We further showed that RhoGDI can actively extract not only inactive, but also active RhoGTPases, which contributes to the spatial regulation of RhoGTPase activity around wounds. In contrast to the textbook model of the RhoGTPase cycle, these results indicate that RhoGDI is more than a passive shuttle for inactive RhoGTPases; instead, it actively contributes to spatiotemporal patterning by removal of active RhoGTPases from the plasma membrane.

P1485/B618

Bnip-2, a Versatile Scaffold in Regulating Small Gtpases.

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Despite being implicated in various physiological and pathological processes, how activity of Rho GTPase is regulated under different cellular contexts remains unclear. In some cells or systems, active Rho could activate or suppress cell morphogenesis and cell migration, raising the possibility that Rho alone is insufficient to exert such effects and that it may require other helper proteins to coordinate its function. For example, generation of force on extracellular matrix (ECM) through actin-myosin contractility and stress fibre is linked to active RhoA. Here, we further report that BNIP-2 that carries a highly conserved BCH domain, serves as a functional scaffold that carries a novel RhoA-interacting motif to exert a clear and effective spatial temporal activation of RhoA during cell morphogenesis. On the other hand, absence of BNIP-2 reduces RhoA activity, force generation on the ECM and focal adhesion dynamics. Functionally, changes in the BNIP-2 expression level could modulate cancer cell spreading and migration, thus providing further insights to the regulation of cell motility and metastasis.

P1486/B619

TC10 GTPase Regulates Matrix Degradation in Breast Tumor Invadopodia.

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Cancer invasion utilizes specialized actin-rich protrusions known as invadopodia. Rho family p21 small GTPases regulate invadopodia functions, including degradation of extracellular matrix through recruitment of membrane-type 1 metalloproteinase (MT1MMP). We are investigating the role of TC10 GTPase as a regulator of MT1MMP function at tumor invadopodia. TC10 GTPase is a member of the Rho family p21 small GTPases. GTPases are molecular switches which cycle between an activated (GTP-bound) state or inactivated (GDP-bound) state and are critical for regulating cell motility and migration. While TC10 has been well-studied in other diseases, it remains understudied in relation to cancer. From our current studies, we demonstrate that TC10 regulates the ability of tumor cells to degrade the extracellular matrix at invadopodia. TC10 controls the vesicular trafficking and MT1MMP localization and its surface presentation at invadopodia, important for matrix degradation. We show directly the spatiotemporal coordination of TC10 activities at invadopodia in breast cancer cells through developing new generations of FRET biosensors for TC10. Our FRET biosensors revealed the dynamics of TC10 activity in tumor cell invadopodia structures. Moreover, our results suggest regulation of TC10 activity by putative upstream regulator GAP to control spatial and temporal locations of MT1MMP function at invadopodia. Importantly, mechanisms driven by TC10 directly impact breast adenocarcinoma invasion, dissemination and metastasis in mice. Our results point to TC10 as a new regulator of tumor metastatic cascade, important for controlling the ability of tumors to efficiently spread to distant sites.

P1487/B620

Crosstalk of ROCK2 with the Dna Repair Machinery Regulates Radioresistance in Cervical Cancer.

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Crosstalk of ROCK2 with the DNA repair machinery regulates radioresistance in cervical cancer

Radiation is the most effective anticancer therapy in cervical cancer. Reportedly high relapse rates are associated with therapy in this cancer. In our study, we have identified ROCK2 a downstream effector of RhoC to contribute to the radioresistance in the tumor cells. ROCK2 increasingly localized to the nuclear compartment post-irradiation. The irradiated cells displayed an enhanced DNA damage repair response mainly of the NHEJ pathway. Overexpression of ROCK2 is shown to confer resistance, while its inhibition caused a reversal of the resistance phenotype in these cells. A transcriptomic analysis revealed that the ROCK2 high cells, live sorted using a novel technique, showed enrichment of genes of the DNA repair, survival and cell cycle pathways. Our data suggests an interaction between ROCK2 and pH2Ax involved in foci formation induced in response to irradiation. Additionally, ROCK2 inhibition resulted in a reduction in the number foci formed by pH2Ax and diminished levels of the MRE11 and RAD50 proteins of the MRN complex, following irradiation. ROCK2 and the DNA repair proteins expression analyzed in clinical sections showed an upregulation. Further, inhibition of ROCK2 in the clinical sample derived cells, in *vitro,* sensitized the tumor cells to radiation. Thus, our data suggests crosstalk of ROCK2 with the

DNA repair proteins modulates radioresistance in cervical cancer and could a potential target for biomarker development.

P1488/B621

GTPase Steering by an Enzymatic Corral.

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Dynamic arrays of actin filaments and myosin-2 ("actomyosin") drive a broad variety of dynamic biological processes ranging from cell division to wound repair. Such arrays are controlled by the Rho GTPases, proteins that exert their effects on actomyosin by stimulating "effector" proteins when in their active (GTP-bound) state. Traditionally, information flow from the Rho GTPases to the cytoskeleton has been viewed as linear, with GTPase activators (GEFs) stimulating a given GTPase, which then activates effectors which, in turn, modify actomyosin. Subsequently, the process is terminated by inactivation of GTPases by inhibitor proteins (GAPs). However, it is becoming apparent that control of actomyosin arrays entails rapid flux of GTPases from the active to inactive stages that is somehow subject to continual modulation via feedback from the actomyosin itself. Here we test a feedback model in which circular, ring-like waves of Rho activity that direct cell wound repair in the Xenopus oocyte model are driven forward by a self-organizing "enzymatic corral" that forms at their trailing edge. Results indicate that 1) trailing edge Rho inactivation depends on an F-actin binding protein Cortactin, 2) Cortactin exerts its effects on Rho activity by serving as a binding site for multiple GAPs (RG1, RG8, and P190RhoGAP), and 3) proper Cortactin and GAP localization/corral formation is F-actin dependent. Cortactin, P190RhoGAP, and RG1/8 localize behind the Rho zone, perfectly situated to facilitate the trailing-edge inactivation. As predicted, Cortactin and the GAPs functionally affect Rho levels. Over-expression of P190RhoGAP or RG1/8 dramatically and selectively diminishes the Rho zone. Expression of a Cortactin truncation missing its SH3 domain (implicated in GAP recruitment) causes decreased GAP localization and increased Rho levels. F-actin assembly, most likely mediated by the GTPase Cdc42, plays a crucial role in corral formation as well. Inhibition of F-actin polymerization via Latrunculin B treatment imparts gaps in the Cortactin, P190RhoGAP and RG1/8 rings, allowing active Rho to "escape" the corral and preventing wound closure. Additionally, recruitment of an actin depolymerization factor (DeActs) upon wounding displaces the F-actin ring outward, away from wound center. This too causes an outward spreading of active Rho and wound healing failure.

P1489/B622

Cdc42 GEF Gef1 Regulates Rho1 Activation During Cytokinesis**.**

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Udo Onwubiko[1], Emma Koory[1], Ahmad Mitoubsi[1], and Maitreyi Das[1] [1]Department of Biochemistry & Cellular and Molecular Biology, University of Tennessee, Knoxville, TN, USA. Email: uonwubik@vols.utk.edu **Abstract** in cytokinesis, key steps ensure that the cytoplasm is properly partitioned, to yield two new, daughter cells after mitosis. We use *Schizosaccharomyces pombe* to understand the coordination of these cytokinetic steps. In *S. pombe*, septum formation is required for furrow formation. During anaphase the conserved GTPase Cdc42 is activated by the Guanine Nucleotide Exchange Factor (GEF) Gef1 to promote recruitment of the septum synthesizing enzyme Bgs1 at the division site and allow timely onset of ring constriction and septum formation.[1] Gef1 is also involved in the spatial organization of proteins along the actomyosin ring thus promoting centripetal furrowing and

septum formation.[2] We investigated the initiation of Bgs1 septum synthesizing activity during cytokinesis. Bgs1 activity requires Rho1 GTPase activity. Rho1 is activated by the essential GEF Rgf3, which localizes to the division site immediately after actomyosin ring assembly[3-5]. Using a mNeonGreen-tagged Rho-probe, we find that Rho1 is activated later, after completion of anaphase B. However, in *gef1Δ* mutants, the Rho-probe localizes prematurely, immediately after ring assembly. Interestingly Rgf3 localization is unchanged in both *gef1*$^{+}$ and *gef1*Δ cells. The p21-activated kinase has been implicated in premature initiation of ring constriction[7]. We find that premature Rho1 activation in *gef1* mutants is partially rescued by the *pak1* mutant *orb2-34*. Our results suggest that Gef1 inhibits Rho1 activation during anaphase at least in-part through Pak1. Thus, while Gef1 promotes Bgs1 recruitment during anaphase, it prevents early Bgs1 activity—and subsequent septum formation—likely by inhibiting Rho1 until chromosome segregation completes. Future studies will probe the mechanistic details of this regulation.

P1490/B623

ARHGAP17, a Cdc42-Specific GAP, Localizes to Invadopodia and Regulates Their Turnover.

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Cancer cells can invade through tissues and metastasize by forming actin-rich membrane protrusions called invadopodia. The formation of invadopodia involves a dramatic rearrangement of the actin cytoskeleton, a process that is regulated by the Rho family of small GTPases. Rho GTPases cycle between an inactive GDP-bound and an active GTP-bound form. RhoGEFs (guanine nucleotide exchange factors) catalyze the activation of Rho proteins, whereas RhoGAPs (GTPase activating proteins) mediate their inactivation. Since RhoGAPs play a critical role in the termination of signal transduction, mutations in genes encoding RhoGAPs have drastic consequences and underlie several human diseases including cancer. However, compared with RhoGEFs, which have been studied more extensively, there is significantly less known about RhoGAPs, especially regarding their role in cancer progression and metastasis. Here, we have identified a RhoGAP, ARHGAP17, as a potential key regulator of invadopodia in both triple negative breast cancer (SUM159 and MDA-MB-231) and Head and Neck Squamous Cell Carcinoma (UMSCC1 and SCC61) cells. Our results show that ARHGAP17 localizes to invadopodia and negatively regulates their turnover. We also show that, in these cells, ARHGAP17 has GAP specificity for the Rho GTPase Cdc42. Based on our results, we propose that ARHGAP17 is targeted to invadopodia where it promotes invadopodia disassembly by locally inactivating Cdc42.

P1491/B624

Connectivity an alysis of GEF/GTPase Networks in Living Cells.

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Fluorescent biosensors are powerful tools to map the activation of signaling molecules in space and time. However, it has not been possible to quantify how different activation events, visualized at specific times and subcellular positions, affect one another or contribute to cell behavior. Here we demonstrate the use of partial correlation analysis to parse out such relationships from multiplexed biosensor imaging data. We show that these approaches can quantify the fraction of a Rho GTPase signal that results from a particular RhoGEF, the relative contribution a RhoGEF makes to each of the downstream

GTPases it interacts with, and importantly determine these relationships with respect to output, e.g. cell motility vs other downstream functions. First, we produced a new series of biosensors to visualize activation patterns of six diverse Dbl family RhoGEFs. We then focused on a pair of these, Vav2 and Asef, and correlated their activity with edge dynamics. To allow simultaneous imaging, we then red-shifted the emission wavelengths of GTPase biosensors and used these new sensors to image and correlate the activation of GEFs and GTPases concurrently in the same cell. Using partial correlation analysis, we identified when and where the GEF Asef regulates the GTPases Cdc42 and Rac1 to control cell edge dynamics. We found that Asef activity is strongly coupled to cell protrusion dynamics as compared to Vav2. Furthermore, Asef strongly activates Cdc42 signals related to cell edge movement. In contrast, the interaction between Asef and Rac1 is weaker and probably indirect, and not related to edge movement. This approach exemplifies a powerful means to elucidate the real-time connectivity of signal transduction networks.

P1492/B625

Induction of Cortical Excitability in Immature *Xenopus* Oocytes.

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During cytokinesis in amphibian and echinoderm embryos, the cell cortex undergoes a dramatic, cell cycle-regulated change in its dynamics: it spontaneously generates propagating waves of Rho activation and inactivation and F-actin assembly and disassembly. The waves are proposed to result from Rho autoactivation, which is dependent on the Rho Guanine Nucleotide Exchange Factor (GEF) Ect2, coupled with delayed, F-actin-dependent, Rho autoinhibition. We have found that overexpression of a mutant form of Ect2 induces excitability in immature oocytes, which normally do not display cortical excitability. However, this excitability is low level and short-lived. In contrast, co-expression of the mutant Ect2 and a Rho GTPase Activating Protein (GAP) results in cortical excitability that is robust, long-lived, and characterized by remarkable spiral wave trains throughout much of the cortex. Using this model system, we have begun to uncover other potential regulators of cortical excitability and place them in the cycle. Diaphanous related formin 3 (diaph3/mDia2) displays excitable dynamics and appears to be the missing link between Rho activation and F-actin polymerization. An illin, a conserved cytokinetic regulator protein, also displays excitable dynamics in the immature oocyte model and appears to be involved in negative regulation of Rho dynamics. Together, these results indicate that both Rho GEFs and GAPs are needed to ensure a stable, excitable cycle, and also that the oocyte is a powerful model for understanding cortical excitability.

Extracellular Vesicle Signaling

P1493/B626

Centrosome Amplification Promotes Extracellular Vesicles Secretion.

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Centrosome amplification is a recurrent feature of human tumours and enforced centrosome amplification plays a role in tumour initiation and progression. However, centrosome amplification occurs only in a subset of cancer cells and thus, partly due to this heterogeneity, the contribution of centrosome amplification to tumours in unknown. Our recent work uncovered an unexpected function for these abnormalities by altering secretion, suggesting a broader role in tumourigenesis. We found that centrosome amplification is sufficient to induce secretion of small extracellular vesicles secretion (sEVs) in pancreatic cancer cells. This is due to a stress response caused by increased reactive oxygen species (ROS). Blocking ROS in these cells prevents sEVs secretion. Our data indicates that increased ROS prevents efficient lysosome function and degradation of the multivesicular bodies, culminating with increase secretion of sEVs of endocytic origin. Interestingly, sEVs secreted by cells with extra centrosomes robustly activate pancreatic stellate cells, suggesting that they contain different cargoes. SILAC analyses identified several unique proteins that are present in sEVS secreted by cells with amplified centrosomes. We are now investigating the sEVs cargoes that could be responsible for PSC activation. Because activation of pancreatic stellate cells creates a strong fibrotic environment that is characteristic of pancreatic cancer, our data suggests that cancer cells with extra centrosomes could play important roles in the tumour microenvironment.

P1494/B627

Identification of Exosome Associated Factors That Protect Against Aminoglycoside Induced Hair Cell Death.

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Exosomes are cell-derived extracellular vesicles that facilitate cell-cell communication. They range from 50-150 nm in size, possess a lipid bilayer, and contain a distinct combination of proteins, lipids, and nucleic acids. Exosomes can induce functional changes in target cell behavior, either through interaction with cell surface receptors or fusion with the plasma membrane. We have recently shown that exosomes play a role in intercellular communication in the inner ear. Exosomes derived from heat-shocked utricles protect hair cells from aminoglycoside-induced ototoxicity and exosome-associated heat shock 70 kDa protein (HSP70) is required for this pro-survival effect. Given the small yield of exosomes from mouse utricles, we are expanding our studies to include cell line-derived exosomes. Here, we are further investigating the protective role of HSP70 as well as the functions of other exosome-associated proteins involved in exosome targeting and delivery, such as integrins. Whole-organ cultures of utricles from adult mice were used as a model system, and multiple cell lines were analyzed for exosome secretion. Exosomes were isolated via centrifugal ultrafiltration, quantified by nanoparticle tracking analysis, and applied to utricles to investigate their ability to inhibit neomycin-induced hair cell death. Tandem mass spectrometry and Western blot analysis were used to identify and

compare exosome content from inner ear tissue and cell lines. Native exosomes derived from an epithelial cancer cell line (CT26) protected hair cells from neomycin-induced hair cell death in a concentration-dependent manner. Both heat shock and exogenous expression of HSP70 increased the amount of HSP70 in CT26 exosomes. Comparative mass spectrometry analyses showed that CT26-derived exosomes contain multiple members of the HSP70 protein family. Exosomes derived from utricles and CT26 cells contain similar repertoires of integrin family members, which may play a role in targeting exosomes to inner ear cell types. The identification of otoprotective exosomes derived from cell lines enables the development of precision-engineered exosome therapeutics. Compared to inner ear tissue, cell lines can yield large quantities of exosomes and can be subjected to biological manipulation aimed at optimizing exosome content. In addition, analysis of exosome cargo established an inventory of factors that may be required for the pro-survival effect in the inner ear. Using this information, we intend to engineer cell line-derived exosomes to systematically investigate which exosome-associated factors are necessary and sufficient to protect hair cells.

P1495/B628

Exosomes Mediate Sensory Hair Cell Protection in the Inner Ear.

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Hair cells are the mechanosensory receptors of the inner ear, responsible for hearing and balance. Hair cell death and consequent hearing loss are common results of treatment with ototoxic drugs, including the widely-used platinum chemotherapeutics or aminoglycoside antibiotics. Induction of heat shock proteins (HSPs) confers protection against aminoglycoside-induced hair cell death via paracrine signaling that requires extracellular Heat Shock 70 kDa Protein (HSP70). Here we investigated the mechanisms underlying this non-cell-autonomous protective signaling in the inner ear. We found that in response to heat stress, inner ear tissue releases exosomes, nanometer-sized endosomally-derived secretory vesicles that mediate intercellular communication by delivering protein, lipid, and nucleic acid cargo. Unique surface molecules allow exosomes to interact with target cells either by internalization or by signaling through cell surface receptors, and this can alter target cell function and fate. Tandem mass spectrometry analysis showed that exosomes secreted from heat-shocked inner ear tissue carry HSP70, as well as a variety of classical exosome markers and other proteins. Isolated inner ear exosomes were sufficient to improve the survival of hair cells exposed to the ototoxic aminoglycoside antibiotic neomycin, while depletion of exosomes from the extracellular environment or inhibition of exosome biogenesis abolished the protective response commonly induced by heat stress. A conditional knock out mouse model was used to demonstrate that expression of the known receptor for HSP70, Toll-like receptor 4 (TLR4), on sensory hair cells was necessary for the protective effect of exosomes, and a proximity ligation assay showed that exosomal HSP70 interacted with TLR4 on hair cells. Taken together, our results reveal a previously-undescribed mechanism of pro-survival intercellular communication in the inner ear and show that exosomes mediate non-autonomous hair cell survival under ototoxic conditions. These exosomes may hold potential as a new class of therapeutics for hearing loss.

P1496/B629

Fibronectin in Extracellular Vesicles from Hepatocytes Mediates Target Cell Binding.

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Background: Extracellular vesicles (EVs) are emerging as important mediators of intercellular signaling in the healthy and diseased liver. Recently we showed that cell surface integrins mediate the cellular binding of EVs from liver cells such as hepatocytes or hepatic stellate cells. In our quest to identify potential integrin ligands in EVs, we now report the presence of fibronectin (FN1) in hepatocyte EVs and its requirement for EV binding to target cells. Our findings point to a new role for FN1 in mediating binding interactions between hepatocyte EVs and hepatic target cells. We also show that serum FN is, in part, EV-associated. **Methods and Results:** EVs were collected by differential ultracentrifugation of serum-free conditioned medium from mouse AML12 hepatocytes. These EVs, termed EV-Hep, were 50-500nm in diameter as assessed by nanoparticle tracking analysis and were positive by Western blot analysis for EV-associated proteins such as CD63, CD9, and Flotillin-1. Proteomics showed that FN1 was the most abundant protein in EV-Hep. Sequence analysis of EV FN1 confirmed its identity as principally the plasma form of FN1 which is well characterized as a hepatocyte product (as compared to the cell-associated form of FN1 which is produced by numerous cell types). The abundant presence of FN1 in EV-Hep was verified by Western blot and co-immunoprecipitation with EV proteins. After iodixanol isopycnic ultracentrifugation, FN1 co-sedimented with EV-Hep at a buoyant density of approx. 1.15 g/ml. FN1 knockout AML-12 cells (FN1KO) were generated using CRISPR-cas9 and the EVs produced from these cells (EV-Hep^{FN1KO}) were used for functional analysis. Yield and size-range for EV-Hep^{FN1KO} were similar to those of EV-Hep, indicating that FN1 is dispensable for EV biogenesis. Fluorophore-labeled EV-Hep bound to FN1KO cells as efficiently as parental cells whereas the binding of EV-Hep^{FN1KO} to either target cell was highly reduced. These results suggest that vesicular but not cellular FN1 is required for binding of EV-Hep to target hepatocytes. As compared to EV-Hep which we have reported to be anti-fibrogenic for HSC, EV-Hep^{FN1KO} showed reduced binding to HSC and reduced ability to attenuate fibrogenic gene expression (CCN2, collagen 1α1). Finally, FN was also present in EVs from primary mouse hepatocytes, human hepatoma HepG2 cells, and human or mouse serum. **Conclusions:** FN1 is abundantly associated with hepatocyte-derived EVs and is required for EV binding to and downstream signaling in target hepatocytes or HSC. EVs are potentially important vehicles for FN transport intercellularly and in the circulation.

P1497/B630

Analytical Ultracentrifugation and Super-Resolution Microscopy Identify Distinct Populations of Extracellular Vesicles: First Steps Toward Discovery of Signaling EV-omics.

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Autosomal dominant polycystic kidney disease (ADPKD) is characterized by abnormal formation of cysts in the lining of renal tubules. 95% of ADPKD cases are caused by mutations affecting ciliary TRP polycystin channel complex formed by two proteins, PKD1 and PKD2 (Polycystic Kidney Disease 1 and 2). In healthy kidney, the polycystins localize to renal cilia. Mutations that abrogate ciliary localization of PKD2 (yet preserve its channel function) also cause cysts. Beside cilia, PKD2 is also found in other subcellular locations including extracellular vesicles (EVs) of human urine. Whether these PKD2-carrying EVs are of ciliary origin and what role they play in healthy and diseased kidney remains unknown. PKD2

is evolutionary conserved across all members of Eumetazoa. In the model organism *C. elegans*, PKD2 is exclusively expressed in the ciliated male-specific neurons and is trafficked to cilia and EVs. The C. elegans PKD-2 containing-EVs play signaling role in inter-organismal communication. Evolutionary conservation and similarity in cellular localization of PKD2 among *C. elegans* and mammals suggest that their network of molecular interactions is likely to be conserved. We propose that PKD2 plays distinct roles in cilia and ciliary EVs. This work aims to identify PKD2-associated EV proteome, transcriptome, and metabolome of *C. elegans to* understand the role of PKD2-carrying EVs in inter-organismal signaling. We established a pipeline for fluorescent labeling and tracking single cell specific EV cargoes in a living animal using super-resolution microscopy. This innovative approach also enables identification of the specific EVs during biochemical enrichment procedures and allows for streamlined optimization. Our results of buoyant density centrifugations in iodixanol gradients suggest that there are two populations of PKD2-carrying EVs that correspond to the densities of 1.12 and 1.14 g/mL. We are currently characterizing these two distinct populations using transmission electron microscopy and refining our enrichment protocol for protein identification by mass spectrometry, sequencing of their RNA cargoes and metabolome analysis. Identification of molecular mediators of the PKD2 signaling will inform on the interactome of human PKD2 and its function in cilia versus EVs.

Mechanotransduction 1

P1498/B632

Macrophage Primary Cilium Lengthening Inhibits Osteoclastogenesis.

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Osteoporosis is a metabolic bone disease characterized by low bone density and deterioration of bone architecture that ultimately increases the risk of fragility fractures. The physical impairment is compounded by the insufficiency of prophylaxis and treatment options devoid of negative side effects. As a result, there is a need to discover better therapeutic solutions to address this disease. Our lab and others have established the osteocyte primary cilium - a mechanosensing, antenna-like organelle - as a promising pharmaceutical target to exploit the body's natural anabolic response to physical loading to maintain bone health. However, little is known about the potential impact of targeting the primary cilium in relation to osteoclasts, the bone resorbing cell. Thus, the *objective* of this study is to determine the presence or absence of osteoclast primary cilia and determine whether or not the macrophage primary cilium serves a functional role in osteoclastogenesis. We cultured RAW 264.7 cells in DMEM supplemented with 10% FBS and 1% P/S, and added 30 ng/mL of macrophage colony stimulating factor (m-CSF) and 100 ng/mL of receptor activator of nuclear factor-Kappa B ligand (RANKL). We performed double immunocytochemistry to observe the centrosomes, primary cilia, and cortical actin of both macrophages and osteoclasts. Next, we treated one group with 10µM fenoldopam mesylate (a known cilia-lengthening agent) for 16 hours. Cells grew for an additional 4 days prior to fixation or lysis. RNA isolation was performed using TriZol, followed by standard curve RT-qPCR to quantify relative measures of tartrate resistant acid phosphatase and cathepsin K. After five independent stains of undifferentiated RAW 264.7 cells, we counted a total of 484 cells and noted a 49% cilia incidence in macrophages, concluding that these osteoclast precursors do in fact possess primary cilia. However, almost none of the differentiated osteoclasts possessed a primary cilium. In addition, we found that cells treated with fenoldopam had statistically significantly lower expressions of ACP5 and CTSK than the vehicle control. We have concluded that while primary cilia are present on macrophages, they are absent from

osteoclasts themselves. Furthermore, inhibiting macrophage primary cilia resorption downregulates osteoclastogenesis, indicating that resorption may be a necessary step for differentiation to occur. Although it is currently unclear, elucidating the role of primary cilium disassembly will shed light on the process of osteoclast formation and function. Lastly, our findings indicate that the primary cilium as a therapeutic target for bone disease may in fact offer a dual beneficial approach to both promote bone formation and downregulate osteoclast activity.

P1499/B633

Proteomic Identification of Centrin-like Proteins from the Cytoskeleton of the Ciliated Protozoan *Spirostomum*.

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Some species of ciliated protozoa, such as *Vorticella* or *Spirostomum*, undergo very rapid contractions that do not require ATP and are triggered by cytosolic Ca^{2+}. These contractions are mediated by specialized cytoskeletal structures, called spasmonemes or myonemes, which are composed of bundles of thin filaments in close association with membrane-bound Ca^{2+} stores. To better understand the structure and regulation of these contractile assemblies, methods were developed to prepare cytoskeletal residues from *Spirostomum*. Detergent-based methods to isolate the cytoskeletal framework of this large ciliate were initially frustrated by the fragility of the extracted cells. It was found, however, that chemical cross-linking prior to detergent extraction stablized the cytoskeletal structures, while still removing most other cellular contents (confirmed using DIC microscopy). Proteins from pelleted cytoskeletal residues were analyzed by gel electrophoresis and two major protein bands were excised for analysis by mass spectrometry using the published *S. semivirescens* transcriptome data (Hines et al., 2018). An alysis of the mass spectrometric data revealed the presence of three different EF-hand calcium-binding proteins, which were homologous to centrins and *Paramecium* infraciliary lattice proteins. Genes encoding these putative components of *Spirostomum* myonemes are being synthesized for expression in bacteria with the goal of reconstituting this contractile apparatus in *vitro*.

P1500/B634

Dynamic Basal Body-associated Striated Fibers Promote Ciliary Array Organization through Basal Body Coupling and Cortical Interactions.

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Multi-ciliary arrays promote cellular motility and fluid flow using the polarized and coordinated beating of hundreds of motile cilia. Basal bodies (BBs) nucleate and position cilia into longitudinal rows. Cilia beat in an asymmetric pattern and produce forces that are imposed upon BBs and the cell cortex. Even in the face of these forces, BBs maintain their position and polar orientation. To promote BB anchorage and orientation, each BB possesses appendages, including striated fibers (SFs) and bundles of microtubules that establish BB coupling. Mutants resulting in shorter SFs cause BBs to disorient. Here, we show that disoriented BBs in *Tetrahymena* multi-ciliary arrays are reoriented into organized ciliary rows when the length of the SF is restored. This reveals that multi-ciliary arrays possess error correction mechanisms to resolve BB disorientation. EM tomography and super resolution fluorescence microscopy were used to show that BBs within the same longitudinal rows are coupled by interactions between the

SFs and post-ciliary microtubule bundles. In addition, SFs adopt different length states by their shrinkage and growth in response to reduced and elevated ciliary forces, respectively. This promotes dynamic BB coupling and cortical interactions that are responsive to environmental forces. An alogous to vertebrate SFs, *Tetrahymena* SFs are multiprotein macromolecular complexes. They are composed of a network of at least eight protein components, belonging to the SF-assemblin protein family, that exhibit distinct and overlapping localization profiles within the SF structure. Components associated with the base of the SF control both steady state SF length and high ciliary force-induced SF elongation. Thus, the dynamic regulation of SFs promotes BB and cortical interactions to organize and couple cilia within ciliary arrays. This enables efficient generation of hydrodynamic flow to serve cellular and extracellular motility.

P1501/B635

T-cell Priming Is Enhanced by Maturation-dependent Stiffening of the Dendritic Cell Cortex.

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Priming of T-cell responses by dendritic cells (DCs) is essential for protective immunity against pathogen invasion and cancer. T cell activation involves multiple receptor-ligand interactions occurring in concert at a specialized cell-cell contact site termed the immunological synapse (IS). Recently, it has become clear that T cell activation at the IS depends on the application of mechanical forces exerted by the T-cell actin cytoskeleton. Since the DC acts as the substrate for T cell activation, we reasoned that the biophysical properties of the DC cortex are likely to provide important mechanical cues. While conventional imaging approaches have greatly advanced our understanding of T-DC interactions, they do not provide information on the biophysical properties of the cells and their role in relaying mechanical cues at the IS. We therefore turned to atomic force microscopy to measure the cortical stiffness of DCs. We show that as part of the maturation process induced by inflammatory stimuli, DC cortical stiffness is increased 2-3 fold, in an actin cytoskeleton dependent manner. Using pharmacological inhibitors and DCs from KO mice, we identify several actin regulatory pathways downstream of Rho GTPases that govern the maturation-associated modulation of DC stiffness. By manipulating the stiffness of T-cell substrates using stimulatory hydrogels or DCs expressing mutant cytoskeletal proteins, we show that increasing stiffness over the range observed for DC maturation lowers the agonist dose needed to initiate T-cell activation. Thus, DC cortical stiffness is a novel, biophysical costimulatory mechanism that functions in concert with canonical receptor-ligand signaling cues. Taken together, our data reveals that maturation-associated changes in the DC cytoskeleton alter its biophysical properties to create a platform for enhanced mechanotransduction, facilitating T cell priming.

P1502/B636

Actomyosin Contractility Dependent Immunological Sensitivity of Immune Cell Activation.

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Macrophages play an important role in inflammation and immune responses, and their malfunctioning is associated with the progression of chronic human diseases such as cardiovascular disease, obesity, and cancer. By recognizing surrounding physiological condition through various membrane receptors, macrophages can be activated and dramatically changes the functional phenotype to perform various

immunological tasks. There are multiple functional states of macrophage activation induced by various immune pathways, including the most well-known contrasting phenotype, pro-inflammatory (M1) and alternative (M2) activation. Although macrophages are located in various tissues exposed to different physical environments, however, how the extracellular mechanical signals determine their immunological activation remains unclear. Here, we demonstrate that immunological activation of bone marrow-derived macrophage (BMDM) is precisely controlled in response to cellular mechanosensation of substrate compliance. To quantitatively assess the cellular heterogeneity of macrophages, we have developed an automated high-throughput cell phenotyping (HtCP) technology, which enables us to obtain essential cellular information such as morphology and specific protein expression at the single cell level. As the substrate stiffness decreases, BMDMs are poorly spread with decreasing F-actin level and show reduced expression of iNOS and arg-1 in response to immunological activators of M1 and M2 activation pathways. Moreover, nuclear translocation of NF-κB and STAT6, key transcription factors of activation pathways, is controlled by substrate stiffness, and vertical compressive forces, which potentially increases nuclear stresses, also promotes nuclear localization of transcription factors. We also find that protein expression of iNOS and arg-1 induced by macrophage activation is tightly coupled with nuclear translocation of each transcription factors, respectively. In addition, the reduction of actomyosin contractility by using cytoskeletal drugs resulted in a decrease of the level of macrophage activation, especially the M2 activation was more sensitively regulated. These results reveal that actomyosin contractility mediated cell spreading can act as a common immuno-regulatory pathway to determine the immunological sensitivity of macrophage to the physical properties of extracellular microenvironment, providing a new perspective on the relationship between physical cues and immune responses.

P1503/B637

Clot Contraction Drives Structural Redistribution of Platelets, Fibrin and Red Blood Cells through Energy Minimization.

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Blood clot contraction, or the volume shrinkage of the clot, plays a critical role in the restoration of blood flow past otherwise obstructive thrombi. Contraction is driven by platelet actomyosin forces and results in the compression of erythrocytes into a tessellated network in the core of the clot, leading to the terminology of polyhedrocytes. The aim of this work was to determine the mechanism driving the redistribution of components of the clot, fibrin and platelets on the exterior and erythrocytes in the interior. Through the use of histology and scanning electron microscopy, we quantified the redistribution of platelets and fibrin to the exterior and erythrocytes into the core of the clot in a time dependent manner during clot contraction. Coupling experimental results with a poroelastic computational model of contracting clots allowed us to discover why this segregated distribution was more favorable than a homogenous distribution. If the clot had a homogenous distribution, it would reach a mechanical steady state immediately. However, as platelets begin to exert contractile forces, if segregation occurs this results in the compression of erythrocytes into the core. As erythrocytes are compressed, they stiffen which effectively results in the platelets generating more contractile forces, furthering the compression of erythrocytes and redistribution of the clot components. The result is a lower energy for the overall system when compared to a homogenous clot distribution, revealing that

energy minimization drives the structural redistribution of components of contracting clots. This conclusion has important clinical implications, since we have previously shown that patients with thrombotic conditions, such as ischemic stroke and venous thromboembolism, have impaired clot contraction compared to healthy subjects, pointing to a potential role of contraction in the obstructiveness and embologenicity of thrombi. Furthermore, the structural redistribution of contracting clots observed here provides a potential basis for the decrease in efficacy of thrombolytic treatment with increasing time following symptom onset and has implications for the development of novel thrombolytics.

P1504/B638

Mechanical Coupling of β_2 Integrins to the Actin Cytoskeleton by a Mechanosensitive Molecular Clutch Drives Complement-mediated Phagocytosis.

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αMβ2 and αXβ2 integrins (complement receptors 3 and 4) are major receptors for phagocytosis of many pathogens and participate in clearance of dead cells and tumor cells. Other integrins mediate cell migration, where their activity and functions are mechanically linked to actin dynamics through focal adhesions (FAs). We tested the hypothesis that mechanical coupling of β2 integrins to a dynamic actin cytoskeleton via FA proteins is essential during phagocytosis for particle binding, outside-in signaling, and engulfment. We used live-cell imaging approaches to characterize actin dynamics, probe the role of key FA proteins, and examine the force generation and stiffness responses of macrophages during integrin-mediated phagocytosis of complement-opsonized target particles. Particle internalization was mediated by formation of Arp2/3-dependent actin protrusions that wrapped around the particle in a formin-dependent manner. Small focal complex-like adhesions formed in the phagocytic cup that contained β2 integrins, vinculin, α-actinin and zyxin, as well as tyrosine-phosphorylated paxillin, FAK and Syk. Perturbation of talin and Syk showed that a talin-mediated link between integrin and actin and tyrosine kinase-mediated recruitment of vinculin mediate force transmission to target particles to promote efficient phagocytosis. Altering target mechanical properties demonstrated more efficient integrin-mediated phagocytosis of stiffer targets. Thus, professional phagocytes co-opt the cell migration machinery, using tyrosine kinase signaling to build a mechanosensitive, talin- and vinculin-mediated molecular clutch, which couples integrins to cytoskeletal forces to drive particle engulfment.

P1505/B639

The C-terminal Domain of Talin Forms a Force-responsive, Directional Bond to F-actin.

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Integrin-based adhesion complexes are dynamic regulators of force transduction between the actomyosin cytoskeleton and the surrounding extracellular matrix. The linkage between integrins and filamentous actin is largely mediated by talin, a 270 KDa adapter protein that also recruits additional actin-binding proteins to assemble the focal adhesion. Force transmission through talin is thought to be initiated by F-actin binding to talin's C-terminal actin binding site (ABS3). Although ABS3 has been characterized in bulk biochemical and cell biological assays, very little is known about how it binds to F-actin under load. We used a single-molecule optical trap assay to study the force-dependent kinetics ABS3 from talin 1 binding to F-actin. We find that ABS3 forms a catch bond to actin when force is applied towards the pointed end of the actin filament, with binding lifetimes more than 100-fold longer than

when force is applied towards the barbed end. Long-lived bonds to F-actin require the ABS3 C-terminal dimerization motif, whose cleavage has been reported to regulate focal adhesion turnover. These results support a mechanism in which the C-terminal actin binding domain of talin preferentially binds and orients actin filaments with barbed ends facing the cell periphery, thus reinforcing F-actin alignment in the lamellipodium and localizing the formation of nascent adhesions.

P1506/B640

Mechanosensitivity Mechanisms of the Lim Domain Protein Testin.

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Mechanical signals generated by the cellular environment influence a wide variety of cellular processes including proliferation, differentiation and migration. The majority of mechanical interactions in cells are mediated by the actin cytoskeleton, which can not only generate and transmit forces, but also convert mechanical signals into biochemical signals in a process called mechanotransduction. Previously, proteins in the LIM family such as zyxin and paxillin, have been shown to be mechanosensitive by localizing to stochastic stress fiber tearing events in the cytoskeleton. The mechanisms underlying this behavior, however, remain unknown. To address this question, we investigated the potential mechanosensitive properties of another multimodular LIM domain protein, the tumor suppressor testin. Testin consists of a CR domain, a PET domain and 3 LIM domains. Using focused laser light, we can induce damage and strain along stress fibers in human fibroblasts leading to recruitment of LIM proteins such as zyxin. When full length testin is overexpressed, it is distributed throughout the cytoplasm and does not localize to sites of strain. When a truncated form consisting of the N-terminal domains is expressed, it localizes to actin stress fibers but does not recognize sites of strain either. When a truncated form of the protein consisting of just the C-terminal LIM domains is expressed, however, it localizes to focal adhesions and will recognize sites of strain. Interestingly, only stress fibers that are not fully severed show recruitment of testin's LIM domains suggesting that these domains recognize a strain-induced conformational change in the actin filaments and not free barbed ends. Additional truncations reveal that the first LIM domain is required for mechanosensitivity. Furthermore, we found that various phosphomutants of testin can drive the full-length protein to focal adhesions but that only a subset of them become mechanosensitive. Specifically, testin becomes mechanosensitive when tyrosines 72 and 111, located between the CR and PET domains, and in the PET domain respectively, are mutated to alanine. Surprisingly, tyrosines 237, 251 and 288 in the first LIM domain, do not affect testin's mechanosensitivity when mutated to alanine. Together, our results indicate a potential role of the N-terminal tyrosines 72 and 111 in regulating the conformation of testin, thereby controlling the mechanosensitivity of the C-terminal LIM domains and their ability to recognize local strain in actin filaments. We speculate that similar mechanisms could be at play in regulating the mechanosensitive behavior of other members of the LIM protein family.

P1507/B641

Effects of Environmental Modifications on *Dictyostelium* Adhesion and Mechanosensation.

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Dictyostelium discoideum is a social amoeba used as a model organism to study directed cell migration, including in response to mechanical stimuli. When introduced to a brief shear flow stimulus *D. discoideum* cells have rapid and transient activation of a number of signal transduction pathways, as

well as actin polymerization at the cortex, although how the cells initially sense the mechanical stimulus is unclear. Currently, there is no known information as to how adhesion affects the response of *D. discoideum* to mechanical stimuli. The question we are addressing is whether a change in adhesion will make a difference in the ability of *D. discoideum* cells to sense mechanical stimuli. Due to a lack of genes coding for integrin, these cells can adhere non-specifically to a variety of surfaces. Sugars and amino acids have been previously shown to inhibit adhesion of *D. discoideum*. Thus, we tested whether glucose, glycine, or a combination of the two treatments affect mechanosensation. Only addition of glucose or a combination of glucose and glycine, but not glycine alone, appeared to lower cell adhesion. However, glucose treatment did not affect the response to a brief, 2 sec stimulation with shear flow, measured by actin polymerization or recruitment of Ras-binding domain as a marker of signal transduction network activation. Future work will focus on testing whether a combination of glucose and glycine, which has the largest effect on random migration of the cells compared to other treatments tested, affects mechanosensation. This study will help us understand whether integrin-independent adhesion plays a role in the cell's ability to detect a mechanical cue and convert it to a biochemical response.

P1508/B642

Effects of Surface Composition on *Dictyostelium* Adhesion and Mechanosensation.

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Highly motile cells of the Dictyostelium discoideum social amoeba are commonly used as a model system for the study of directed cell migration. Mechanical cues, such as shear flow, can induce directed migration of various cells, including Dictyostelium; however, molecular mechanisms that allow cells to sense mechanical cues are poorly understood. Although integrins have been implicated in mechanosensation, Dictyostelium lacks integrins and attaches to substrate in large part due to non-specific interactions mediated by Van der Waals forces. The purpose of this study was to test whether reducing integrin-independent adhesion of cells to the surface would affect Dictyostelium mechanoresponse. To reduce adhesion of cells we used bovine serum albumin (BSA), which has non-specific binding domains that can interfere with electrical charge interactions that bind the cell to the surface. We evaluated cell mechanosensation by measuring actin polymerization in response to very brief, 2 sec, exposure to shear flow on the surfaces coated with various concentrations of BSA. Mechanical stimulation response of Dictyostelium cells grown on a bacterial lawn, which are known to produce a robust response in this assay, was the same on BSA compared to control coating. Interestingly, when we tested adhesion of bacterially-grown cells, there was no significant difference on BSA compared to the control surface, in contrast to previously published literature. Indeed, standard axenically grown cells on BSA-coated surface showed a significant decrease in adhesion and a corresponding increase in velocity during random migration. These results suggest that cells in different stages of Dictyostelium growth and development respond differently on surfaces of differing chemical compositions. Efforts are currently underway to find surface modifications that reduce attachment of bacterially-grown cells to allow for examination of their mechanoresponse under reduced adhesion conditions.

P1509/B643

Mapping the Biochemical Interactions of the Mechanoresponsive Contractility Controller.

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Every biological process, ranging from cell migration to embryogenesis, relies on the cell's ability to adapt to changing mechanical environments. By studying the model shape change process cytokinesis in *Dictyostelium*, we find that the cell is a finely tuned control system, with proteins that modulate their behavior in response to mechanical and biochemical signals (Kee *et al*, 2012; Srivastava *et al*, 2015). We are now unraveling the biochemical interactions that allow force propagation through the cortical network in the contractility controller. We used a proteomics approach to identify direct interactors of two key nodes of the control system, actin crosslinker cortexillin I and scaffolding protein IQGAP2. This analysis identified mechanoenzyme myosin II as a biochemical interactor of both cortexillin I and IQGAP2. We find that the cooperative mechanoaccumulation of myosin II and cortexillin I is potentially due to their direct interaction. IQGAP1, in turn, competes with IQGAP2 to bind myosin II and cortexillin I, thus negatively regulating contractility. Our data also suggest that multi-protein complexes (mechanoresponsive contractility kits, MCKs) are pre-formed in the cytoplasm, primed for activation by chemical or mechanical stimuli to engage with the cytoskeletal network (Kothari *et al*, 2019). In addition, we find a few unusual interactors by mass spectrometry analysis that we previously identified in genetic suppressor screens, providing greater evidence for their function in the network. In particular, we are now investigating the role of methylmalonyl-semialdehyde dehydrogenase (mmsdh), an enzyme that catalyzes a step of the valine degradation pathway. Mmsdh overexpression was initially shown to rescue the growth defect and localization of an assembly-deficient myosin II mutant (Ren *et al*, 2014). Using Fluorescence Cross-Correlation Spectroscopy (FCCS) to measure apparent in *vivo* K_D values, we have found that both myosin II and cortexillin I interact with mmsdh in live cells. Using the contractility controller as a model, we are identifying a quantitative interaction map, complete with new interactions, that is uncovering new biochemistry associated with the mechanobiome.

P1510/B644

Polarity Signaling Ensures Epidermal Homeostasis by Coupling Cellular Mechanics and Genomic Integrity.

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Epithelial homeostasis depends on an elegant equilibrium between progenitor self-renewal and differentiation. This process was thought to primarily be mediated by the orientation of mitotic spindles, but there is growing evidences that other mechanisms contribute. Here, by combining genetic ablation, live cell imaging, atomic force microscopy and mechanochemical reconstitution, we describe a novel function of mammalian Par3 in controling epidermal homeostasis. Inactivation of Par3 impairs actomyosin contractility and viscoelastic properties of keratinocytes, leading to diverse mitotic aberrancies. These mitotic failures trigger aneuploidy, mitosis-dependent DNA damage responses and p53 stabilization that eventually fuel premature differentiation. We unveiled a novel mechanical signaling network in which Par3 regulates RhoA activity and p190GAP expression in keratinocytes. Remarkably, reconstituting myosin activity was not only sufficient to rescue mitotic aberrancies and genome integrity in *vitro* but could also normalize the increased suprabrasal sorting behavior of $Par3^{KO}$ keratinocytes in a stratification model. Collectively, this study deciphers a novel role of Par3

independent of spindle orientation, that couples genome integrity and epidermal fate decisions via the regulation of keratinocyte mechanics.

P1511/B645

An Asymmetric Mechanoresponse at Cadherin Junctions Ensures Epithelial Integrity during Mitotic Rounding.

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Epithelia are continuously self-renewed, but how epithelial integrity is maintained while cells undergo dramatic shape changes during mitosis is not fully understood. Here, we show that mitotic entry coincides with an increase in tensile forces on adhesions between mitotic cells and their neighbours. By physically confining epithelia in height, we demonstrate that the increase in junctional tension is a direct consequence of mitotic cell rounding. To withstand intercellular forces, cadherin-based cell-cell adhesions can trigger an adaptive response to reinforce junctions through recruitment of the actin binding protein Vinculin. Surprisingly, we find that Vinculin is not recruited to cadherin in the mitotic cells but only in their neighbours, resulting in asymmetry in the composition of cadherin junctions. When junctional Vinculin recruitment is inhibited in mitotic neighbours, this results in junctional breakage and a weakened epithelial barrier upon mitotic entry. Conversely, preventing junctional release of Vinculin in mitotic cells attenuate the ability of cells to round up in mitosis. Our data thus identify an asymmetric mechanoresponse at cadherin adhesions during mitosis, which is essential to maintain epithelial integrity while at the same time enable the shape changes of mitotic cells.

P1512/B646

ERK Activation Waves Mediated by Intercellular Mechanical Forces Underlie Long-range Guidance for Collective Cell Migration.

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Collective cell migration is a fundamental process involved in many biological phenomena including epithelial wound healing and embryonic development. During collective cell migration of Madin-Darby canine kidney (MDCK) cells, activation of extracellular signal-regulated kinase (ERK) is propagated as multiple waves from leader cells to follower cells, which directs migration of the follower cells. However, the mechanism underlying the intercellular propagation of ERK activation remains elusive. By combining Förster resonance energy transfer (FRET) biosensor imaging and the use of optogenetic and drug-inducible dimerizers, here we report that intercellular force transmission mediates ERK activation waves. Correlation-based image analysis showed that the follower cells collectively move forward by repetitive cycle of extension and shrinkage, and that the cell deformation waves are propagated as the ERK activation waves. Cross-correlation analysis revealed that the cell deformation waves slightly precede the ERK activation waves; i.e., a cell is first extended, followed by ERK activation, and then the cell starts shrinking. Consistent with this temporal sequence, passive stretching of cells on an elastic chamber results in ERK activation, and optogenetic activation of ERK triggers cell contraction. Given the tight physical connection between the epithelial monolayer cells, we speculated that the ERK-induced cell contraction should lead to mechanical stretch of adjacent cells, by which ERK activation is propagated to the adjacent cells. As anticipated, induction of cell contraction by drug-induced Rho GEF

activation system results in the ERK activation in the neighboring cells. Furthermore, disruption of adherence junctions by the knockout of α-catenin severely impairs the ERK activation waves, confirming that intercellular transmission of mechanical force mediates ERK activation waves. Collectively, we have shown that ERK activation waves are mediated by intercellular force transmission, which underlies long-range transmission of directional cues for collective cell migration.

P1513/B647

Vinculin-mediated Mechanocoupling during Epithelial Sheet Expansion.

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Tissue expansion and remodeling, which underlie many fundamental morphogenetic processes, are thought to be driven by the generation and transmission of actomyosin forces. However, the interplay of adhesion structures and cell-generated forces remains poorly understood. As actomyosin forces are transmitted through both focal adhesions (FAs) and adherens junctions (AJs), we probed the mechanical loading of vinculin, which is known to localize to both adhesions and serve as an actin cross-linking protein, to determine if vinculin is differentially loaded in these compartments. Using a 2D expansion assay and a previously developed Förster resonance energy transfer-based vinculin tension sensor (VinTS), we characterized vinculin load in all three compartments of two epithelial cell lines, one that forms prominent lamellipodia and expands quickly and another that forms pluricellular actin belts and expands slowly. While vinculin load at FAs was similar between the cell lines, we found vinculin was loaded at AJs and throughout a cytosolic (non-stress fiber) actin network only in the fast-expanding cell line. Because a well-defined actin structure was not visible using traditional light microsopy, we confirmed that vinculin was acting as a cross-linker by using fluorescence recovery after photobleaching (FRAP). To determine if vinculin loading was specific to actomyosin contractility, we implemented an actin-binding mutation (I997A) and found VinTS loads were reduced. Additionally, the I997A mutation increased vinculin's turnover in the cytosol based on FRAP measurements. As large mechanical forces in the cytosolic (non-stress fiber) actin network were unexpected, we developed and employed a novel FRET-based tension sensor that acts as a synthetic actin cross-linker. This sensor reported large forces in lamellipodia-based migration, corroborating our findings in VinTS. When cells were plated individually, we discovered that VinTS became unloaded in the cytosol. In total, this work suggests that AJ formation enables the formation of a loaded cytosolic actin network, and we hypothesize this network is important for lamellipodia formation to mediate fast expansion.

P1514/B648

Elevated Extracellular Fluid Viscosity Stimulates Migration of Metastatic Cancer Cells.

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While the significance of the stiffness of the extracellular microenvironment has been well-studied, the role of others physical factors, such as fluid viscosity, are less understood. The viscosities of biological fluids span orders of magnitude, and due to lymph circulation, disease development, and fluctuations in protein secretion, cells directly in contact with mucus, extracellular fluid (ECF), and saliva are often subjected to variations in viscosity. In the context of cancer, leaky vasculature and matrix degradation within the tumor microenvironment lead to high local concentrations of plasma proteins and soluble collagen that could increase ECF viscosity. Moreover, mucins, the large, heavily-glycosylated extracellular proteins responsible for the high viscosity of mucus and saliva, are overexpressed in many

malignancies. In this study, the cellular response to increased viscosity is characterized, especially in terms of tumor progression and metastasis, and a mechanism for this response is proposed. To simulate elevated ECF viscosity, an inert thickening agent, is added to culture medium to increase viscosity up to a maximum of 11,000 times that of water at low shear. Strikingly, human breast cancer cells MDA-MB-231 moved ~2-fold faster in viscous medium for at least 24 hours. In addition to single cell tracking, this result was confirmed using Transwell migration and invasion assays, which demonstrated a similar ~2 fold increase in the fraction of cells passing through a membrane of micron-sized pores. Notably, other types of cancer cells tested also exhibited similar behaviors. This finding is the opposite of what has been reported in neutrophils, which typically exhibit amoeboid rather than mesenchymal migration, suggesting that high viscosity might stall immune mobilization but accelerate metastasis. High-speed timelapse microscopy revealed that an expansion in cell spread area begins instantaneously upon addition of viscous medium. This expansion, which plateaus at approximately 150% and is reversible upon replacement of regular medium, is associated with explosive growth and remodeling of focal adhesions. Drug treatment has shed light on parts of the mechanism of this response, showing that the elevated cell motility and cell spread area are integrin- and actin-dependent.

P1515/B649

Uniaxial Contraction Sustains Cell-generated an terior 3D Matrix Prestress to Initiate and Maintain a 3D Mesenchymal Cell Migration Cycle.

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During cell migration, cytoskeletal contractile forces are transmitted to the microenvironment through integrin-based focal adhesions in a process known as mechanotransduction, which provides a bidirectional pathway for mechanical information to pass to and from the extracellular matrix (ECM). This process has been classically characterized on two-dimensional, flat surfaces, yet it remains unclear how these processes occur within a three-dimensional (3D) microenvironment and how they are incorporated into 3D migration. Here we have uncovered a unique contractile mechanism used by fibroblasts and mesenchymal cancer cells (HT-1080s) to migrate efficiently through 3D collagen gels. An alysis of ECM deformations during 3D cell spreading suggests that 3D cell polarization is highly force-dependent, with cells establishing an unbalanced matrix prestress: Fibroblasts protrude, polarize and initiate migration in the direction of highest ECM deformation. This matrix prestress is maintained through a unique contraction where cells locally pinch the matrix in a uniaxial fashion immediately behind the leading edge, which is concealed by the large absolute ECM deformations but is revealed by instantaneous kinetic analysis. We find this uniaxial contraction (UAC) occurs prior to leading edge protrusion and coordinates a distinct 3D migration cycle that varies between cell types. Local matrix severing using two-photon ablation to disrupt matrix prestress, as well as local contractile inhibition to ablate a uniaxial contraction, both halt forward migration, suggesting both mechanisms are required for mesenchymal migration and may represent a mechanosensing mechanism. An alysis of ECM deformations suggest that fibroblasts transmit large forces to the microenvironment ~3.8-fold higher than MDA-MD-231 cells. We find that epithelial cancer cells (MDA-MB-231) rarely demonstrate a sustained matrix prestress or a uniaxial contraction; however, MDA cell lines established from secondary brain tumors that demonstrate higher myosin IIB expression, but not from secondary bone tumors, demonstrate both mesenchymal phenotypes. Moreover, analysis of integrin ligation and myosin II (A

and B) expression suggests higher expression of both increases a cell's mesenchymal characteristics. Fibroblasts lacking myosin IIA, or if integrin ligation has been reduced, lack a mesenchymal phenotype, while overexpression of either myosin IIA or IIB increases the UAC phenotype We propose that mesenchymal cells can sense ECM stiffness in 3D, generating their own matrix prestress in a myosin IIA- and integrin-dependent manner to migrate. They do so through continued uniaxial contractions that maintain a 3D migration cycle.

P1516/B650

Extracellular Fluid Viscosity Enhances Cell Motility and Invasion.

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Cell migration is critical for many biological processes, including embryonic development, immune cell mobilization and wound healing, among others. In response to both chemical and mechanical cues, cells can move in a directional manner. While migration of normal cells is an important part of homeostasis, motility of diseased cells, especially cancer cells, can lead to undesired consequences, such as metastasis. Cell migration is a complex and highly regulated process. In vivo, motile cells move through the extracellular matrix while immersed in the extracellular fluid (ECF). It is known that the presence of macromolecules such as mucins can increase ECF viscosity, but the effect of viscosity on cell migration is poorly understood. In this study, we investigate how cell migration changes when immersed in the fluid of various viscosity values. Timelapse microscopy was used to record cellular migration in culture medium supplemented with different polymers to adjust viscosity and osmolarity, and cell tracking software was used to analyze the average cell speeds. Here we report evidence that viscous ECF enhances cell motility in both metastatic breast cancer cells MDA-MB-231 and NIH-3T3 fibroblast cells in a graded and sustained manner. For example, in 2D, MDA-MB-231 cells move nearly twice as fast when immersed in medium with a viscosity of ~1000cP. Transwell migration assay showed that viscosity also enhances the cell invasion through confined spaces. Moreover, the increase in migration is viscosity-dependent but osmolarity-independent. Autocorrelation analysis of the trajectories of migrating cells revealed viscosity does not change the migration persistence. Having established that viscosity enhances cell motility and cell invasion, we identified that cells can sense and respond to ECF viscosity by cell spreading. Because viscosity is observed to be significantly higher in tumors due to the leaky vasculature and overexpression of mucins, viscosity-sensing and enhanced motility could play a critical role in tumor progression.

P1517/B651

Plectin Linkages Aid in Moving the Nucleus through Cross-linked 3d Matrices.

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Plectin linkages aid in moving the nucleus through cross-linked 3D matrices Pragati Chengappa[1], Samir Jambhekar[1], Gerhard Wiche[2], and Ryan J. Petrie[1] [1]Department of Biology, Drexel University, Philadelphia, PA [2]University of Vienna, Vienna, Austria Fibroblast migration through three-dimensional (3D) matrices is essential for wound healing and tissue homeostasis. However, the movement of the nucleus is a barrier to cell migration through 3D matrix environments due to its size and rigidity. To overcome this, fibroblasts migrating through cross-linked matrices, such as 3D cell-derived matrix (CDM) and mammalian dermis, use the power of actomyosin contractility to pull the nucleus forward and

maintain rapid 3D migration. The forward movement of the nucleus raises the hydraulic pressure in the front of the nucleus and generates high-pressure protrusions called lobopodia. The nucleus is wrapped by a basket of vimentin intermediate filament which aids in pulling the nucleus through the matrix and generating compartmentalized pressure. Currently, it is not clear how the force generated by actomyosin contractility is connected to the vimentin basket to pull the nucleus through the matrix. Plectin is a cytolinker protein that can cross-link vimentin with other cytoskeleton filaments to arrange and maintain the cytoarchitecture during processes such as cell adhesion and migration. Our work tests the hypothesis that plectin links vimentin and actomyosin filaments to connect the myosin II-generated forces to the nucleus to help pull it through the tight spaces in cross-linked 3D matrices. We found that actin and vimentin filaments are closely associated (often within 10 nm or less) in the cytoplasm of primary human dermal fibroblasts migrating on 2D glass. Both plectin expression and myosin II activity were required to maintain these actin-vimentin interactions in the cytoplasm. These results suggest that plectin mediates actin-vimentin interactions in fibroblasts in a myosin II-dependent fashion. Interestingly, plectin knockdown cells exhibit increased velocity on 2D glass. However, plectin knockdown cells in 3D CDM had significantly reduced hydraulic pressure and vimentin filaments around the nucleus, indicating that the nuclear piston machinery was dismantled. Together, these data suggest that plectin-mediated linkages between actin and vimentin filaments are required for moving the nucleus through 3D CDM, but slow down 2D fibroblast migration. Further, myosin II contractility may be required to maintain these linkages while it is driving nuclear and whole cell movement through physiological 3D cross-linked matrices.

P1518/B652

Microtubule Acetylation Promotes Rigidity Sensing and Mechanosensitive Migration of Astrocytes.

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Cell adhesion to the extracellular matrix occurs through integrin-mediated focal adhesions, which sense the mechanical properties of the substrate and impact cellular functions such as cell migration. Mechanotransduction at focal adhesions affects actin dynamics resulting in migration of cells. Although microtubules are key players in cell adhesion and migration, their role in mechanotransduction still remains unstudied. Using a combination of microfabrication methods, biophysical approaches and imaging techniques such as hydrogel patterning, traction force microscopy and electron microscopy, we show that substrate rigidity affects microtubule acetylation through β_1 integrin signalling in astrocytes but not in glioblastoma cells. Moreover, αTAT1, the enzyme responsible for microtubule acetylation, is required for the mechanosensitive migration of astrocytes. αTAT1 also reorganizes the actomyosin network, increases traction force generation and promotes cell migration on stiff substrates. Our results suggest a novel feedback mechanism involving a crosstalk between microtubules and actin in mechanotransduction at focal adhesions whereby, cells sense the rigidity of the substrate through integrin-mediated adhesions, modulate their levels of microtubule acetylation, which then controls the actomyosin cytoskeleton, force transmission on the substrate and promotes cell migration.

P1519/B653

Cell Matrix Invasion Requires Non-Muscle Myosin 2A/B Polarity and Nuclear-force Coupling Generated from Adhesion Sites.

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Dynamic cell polarisation is observed during cell invasion of 3D matrices. In this study we set out to identify the parameters required for invasive cell migration in 3D environments. Transmembrane-spanning integrin adhesion complexes (IACs) allow the cells to respond to changes in the extracellular environment. IACs are well characterised in cells plated on 2D surfaces, yet much less is known about them in cells embedded in 3D matrices. We establish a BioID-based technique to compare the composition of IACs of invasive breast cancer cells in 2D versus 3D matrices and measure 3D IAC dynamics of endogenous (knock-in) proteins. We identify a novel interaction complex consisting of N-WASP/WIP -> β-PIX -> Myosin-18A (M18A), which is enriched in 3D adhesive sites. Depletion of β-PIX or M18A abolishes cancer cell invasion, without negatively affecting matrix degradation or protrusion formation in collagen matrices. Instead, β-PIX/M18A drive the polarised recruitment of non-muscle Myosin 2A (NM2A) to the tips of protrusions. This recruitment of NM2A is required for the creation of a NM2A/B gradient, which assembles from protrusion to the nucleus. A NM2A/B gradient will provide the actomyosin network with differing catalytic properties of the NM2 isoforms. NM2A is known to have faster motor activity than NM2B, whereas NM2B has a higher affinity for ADP, reflecting longer high affinity attachment to actin filaments. The NM2A/B gradient couples protrusion assembly with nuclear movement, enabling effective invasive migration and polarisation. These findings suggest that actomyosin mediated nuclear-force coupling through a NM2 isoform gradient is a crucial mechanism required for invasive cell migration through 3D matrix.

P1520/B654

Nuclear Pressure and Movement Govern 3D Motility.

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Fibroblast migration through three-dimensional (3D) matrix environments is essential for normal wound healing. During 3D migration, cell morphology and the mechanism of migration are largely dictated by the interactions of the cell with its local microenvironment, including the ability of a cell to squeeze its bulky rigid nucleus through narrow openings. To efficiently translocate their nuclei through the small pores within heavily cross-linked matrices, fibroblasts use actomyosin contractility to pull their nuclei forward like a piston to generate compartmentalized pressure and switch from a lamellipodia- to a lobopodia-based migration. However, it remains unclear how this nuclear piston mechanism is activated in response to specific cell-matrix interactions. Recently, it was shown that the nuclear envelope can transiently rupture in cells moving in 3D environments, suggesting mechanical stress on the nucleus can lead to a dramatic increase in pressure inside the nucleus. Here, we test the hypothesis that cells moving through cross-linked 3D matrices activate the nuclear piston mechanism in response to increased resistance and mechanical stress on the nucleus. Mechanical stress on the nucleus was indirectly measured by quantifying changes to nuclear morphology and intranuclear pressure. Our results show cells moving from the 2D surface to the interior of 3D matrices can dramatically elongate their nuclei without activating the piston mechanism. Since physically deforming the nucleus was not sufficient to

trigger the nuclear piston, we next measured changes in nuclear pressure in cells immediately before and after piston activation. Nuclear pressure was not only compartmentalized and distinct from cytoplasmic pressure, but also increased upon piston activation, suggesting the nucleus is under elevated mechanical stress when the piston is on. Next, we investigated how nuclear pressure was controlled in dermal fibroblasts. We determined myosin II activity and intact nucleoskeleton-cytoskeleton connections are required for the increase in nuclear pressure during piston activation. Overall, our current model of piston activation consists of three distinct steps: 1) the nucleus becomes stuck when migrating in 3D matrix, 2) tension on nesprin linkages increase nuclear pressure and 3) initiation of a signaling cascade within the cytoplasm triggers the actomyosin contractility required to pull the nucleus forward. Future work will determine the temporal coordination of pressure changes inside the nucleus and cytoplasm during piston activation.

P1521/B655

Confinement-induced DNA Damage Causes Increased Matrix Degradation and Invasiveness in Breast Cancer Cells.

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During migratory events such as tissue morphogenesis, immune surveillance and cancer invasion, intracellular forces are transmitted to the nucleus. Using as a model the development of tumor xenografts in the mammary duct of mice Lodillinsky et al. (2016) showed that to breach out of the mammary gland ducts, MCF10 ductal carcinoma in situ cells (MCF10.DCIS) degrade the basement membrane, what induced degradation remained unknown. We observed that the nuclei of these cells are less deformed in early tumor stages when compared to later stages, which also displayed increased DNA damage. We previously showed that strong nuclear deformation leads to nuclear envelope (NE) rupture and DNA damage in RPE1 cells. We thus tested whether the increased nuclear deformation and DNA damage observed in the DCIS xenografts contributes to trigger the invasive phenotype acquired by these cells to progress to invasive carcinoma. We observed that DCIS cells embedded in 3D collagen following strong (2µm) but not mild (10µm) confinement exhibited highly dynamic actin protrusions and increased MMP-dependent collagen degradation. DCIS cells confined at 2µm but not 10µm exhibited frequent NE rupture events and increased DNA damage. We then developed an *on-chip* duct assay to reproduce the confined growth observed in intraductal tumor xenografts. Strikingly, as cell density increased in this device, NE rupture and DNA damage increased, likely due to the high migratory activity of the crowded cells in the confining duct. In both DCIS and RPE1 cells DNA damage is associated to NE rupture, so we next sought to identify a potential cytoplasmic factor that would access the exposed nuclear DNA following NE ruptures. TREX1 is an exonuclease shown to attack chromatin bridges that persisted in cytokinesis following rupture of the NE that reformed around them. Strikingly, both transient TREX1 depletion and stable CRISPR KO in DCIS and RPE1 cells nearly abolished DNA damage associated to NE rupture. TREX1 depletion followed by strong confinement also abrogated collagen degradation and invasion in the *on-chip* duct assay. Strikingly, strong confinement of RPE1 and untransformed DCIS cells (MCF10A) triggered a TREX1-dependent DNA damage and cell senescence. Here we describe a causal relationship between cell confinement, NE rupture and TREX1-dependent

DNA damage with specific long term cellular phenotypes: collagen degradation and invasion in DCIS cells and senescence in RPE1 and MCF10A cells. The mechanism of TREX1-dependent DNA damage in confined cells with NE rupture could relate the observation of deformed nuclei and increased DNA damage (both in duct xenograft tumors in mice and in human breast tumors) to the development of an invasive phenotype and thus contribute to the initiation of certain types of breast cancers.

P1522/B656

Mechanical Compression Reprograms Transcription by Increasing Macromolecular Crowding.

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Tens of thousands of biochemical reactions occur simultaneously in the cell, organized at a wide range of length-scales. Small molecules are channeled through metabolic pathways at blistering speeds. However, at higher length-scales, macromolecular complexes, such as those that orchestrate transcription, experience a high degree of mesoscale crowding that approaches the physical limits where jamming will occur. We combined a new genetically encoded nanoparticle technology with microfluidic pressure chambers to reveal that mechanical compression increases molecular crowding in both the nucleus and cytoplasm. Changes to nuclear crowding increased expression of some genes and decreased others. We propose that crowding increases that activity of some promoters by driving assembly of transcriptional machinery through attraction depletion, while other promoters are sensitive to jamming. This differential response of genes to changes in crowding provides a potential physical mechanism to respond to mechanical compressive stress without the need for a canonical signal transduction pathway.

P1523/B657

A Viscoelastic Model Predicting Cell Mechanical Memory through Mechanical Energy Dissipation of the Cell Nucleus.

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Cellular processes are directly impacted by the mechanicals signals from their microenvironment. Some of those signals are directly transmitted from the adhesion complex to the nucleus via the actomyosin apparatus, stressing the organelle and causing reversible and irreversible phenomena. To fully understand the dynamics of this nuclear strain responsible for important mechanoresponsive behaviors, it is crucial to build a model that considers all the nuclear mechanical properties that have been reported to impact nuclear strain. We developed a model integrating the viscoelastic property of the nucleus caused by histone deacetylation and lamins in order to consider the time-dependent contributions of such important nuclear elements. The model managed the contribution of lamin-A,C and lamin-B to the nuclear strain and stress for different ECM stiffnesses as previously shown experimentally by others. Furthermore, the dynamic role of lamins levels is suggested as an explanation of cell mechanical memory through mechanical energy dissipation that induce irreversibility of the nuclear shape. An d finally, the model seems to suggest to experimentally study the kinetics of nuclear deformation to better understand ECM-stiffness-related mechanisms on cell nuclei, as the timescale during which stress is applied is of great relevance in defining the future of a cell.

1

Cytoskeleton-Membrane Interactions

P1524/B658

A Polarized Spectrin Cytoskeleton Stabilizes Multivalent Endothelial Adhesion Receptor Complexes to Facilitate Rolling Adhesion.

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In the early stages of an immune response, multivalent complexes of endothelial adhesion receptors (e.g. clustered selectins) engage leukocytes to orchestrate their trafficking to inflamed tissues. Sufficient stability and density of these receptor complexes must be maintained for efficient trafficking to occur. We demonstrate that a polarized, spectrin cytoskeleton regulates apical actin-filament stability and mediates the frequent immobilization of the abundant transmembrane protein CD44. We used single-particle tracking and super-resolution microscopy to investigate the effects of a stable CD44-spectrin network on the diffusion and distribution of bystander selectin clusters that do not directly associate with the submembrane cytoskeleton. The network restricted selectin mobility and served to stabilize selectin clusters, bolstering transient, multivalent heterocellular interactions. In this way, the spectrin network is proposed to regulate the initial contacts between leukocytes and endothelial cells to ultimately mediate extravasation.

P1525/B659

A Template for Actin Organization at the Leading Edge.

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How local interactions of actin regulators yield large-scale organization of cell shape is not well understood. For example, why does the WAVE complex build lamellipodia, broad sheet-like protrusions that power cell migration, whereas the highly related actin regulator N-WASP forms finger-like actin networks? from in vitro reconstitutions, sheet-like actin structures require spatially organized actin nucleation along a linear structural template. What is the endogenous basis of a template for lamellipodia in living cells? to investigate whether WAVE complex self-organizes into a template that sculpts lamellipodia, we studied its nanoscale organization at the plasma membrane. Our previous work showed that WAVE complex self-associates into small foci following actin depolymerization. At conventional resolution, these appeared to be amorphous blobs, similar to droplet-like phase transitions exhibited by N-WASP. Using super-resolution microscopy, we found the WAVE complex “foci” are actually highly ordered rings, 280nm wide, which can elongate into micron-length spirals. These linear structures indicate that WAVE complex self-assembles into a linear structure that could represent the physical template for lamellipodial formation; actin assembly perpendicular to these linear arrays of WAVE complex could generate the actin sheets that are the basic unit of lamellipodia organization. An alysis of the membrane organization around these rings provided further insight into the role of geometry in cell morphogenesis. These rings localize to necks of membrane invaginations, which are saddle point geometries (positive curvature in one axis and negative curvature in the other axis). This suggests a specific geometric input into WAVE complex’s localization. This saddle preference for the WAVE complex could explain emergent cell behaviors such as the expanding self-straightening behavior

of lamellipodia as well as the ability of endothelial cells to seal transcellular holes. To investigate the molecular mechanism of saddle curvature recognition, we show that the WAVE complex and IRSp53, an inverse BAR domain protein, collaborate together to recognize saddle geometry that neither can recognize on its own. Our work highlights how partnering protein interactions enable complex shape sensing and how feedback between cell shape and actin regulators yields self-organized cell morphogenesis.

P1526/B660

A Computational Method to Quantify Cell Membrane Remodeling and Cortical Localization of Actomyosin Cytoskeletal Components during Cell Migration in Live an imals.

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Active cell migration requires local membrane remodeling, which involves dynamic protrusions and retractions driven by cytoskeletal rearrangements. State of the art two-photon intravital subcellular microscopy (ISMic) enables the acquisition of 4-D (3D in time) movies of interstitial neutrophil migration during immune response in *vivo* with a resolution of a few hundred nanometers and a frame rate of fewer than 10 seconds. Here, we describe a tool to analyze the dynamics of cell migration at the subcellular level and to understand how local actomyosin dynamics and cell membrane remodeling coordinate to drive the cell migration through the complex interstitial extracellular environment in *vivo*. The proposed computational method was implemented by customized MATLAB codes. The cell shape was quantified in a semi 3-D fashion through a level-set “snake” algorithm, which took into consideration both image brightness gradient and cell boundary smoothness. The codes were applied to the 2-D max z-projections of the segmented 3-D cell voxels to identify reasonable cell boundary contours. At each frame, the cell boundary was defined by consecutive boundary points at subpixel resolution, which enabled the calculation of local boundary curvatures. The local boundary motion from one frame to the next was identified by tracking the boundary points according to the minimum displacement criterion. For each boundary point, a box of a selected depth was defined, and the average intensity of cell voxels projected in that box for the non-muscle myosin IIA (NMIIA)-GFP or LifeAct-GFP acquired signals. This allowed the correlation between the actomyosin complex along the cell cortex and local cell membrane remodeling. Using this tool and the associated statistical analyses, we found that NMIIA was recruited not only to the retracting rear of migrating neutrophils but also at the leading edge. NMIIA localization positively correlated with the local cell boundary curvature and membrane retraction, but not protruding activity. Notably, this quantitatively defined phenotype was not recapitulated in standard in *vitro* assays for neutrophil migration where NMIIA is mostly concentrated at the retracting rear of the cell.

P1527/B661

Regulation of Septin Architecture and Function by the Lkb1-Like Kinase Elm1.

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The septins are a highly conserved family of GTP-binding proteins that assemble into heterooligimeric filaments in eukaryotic cells. These filaments can further organize into higher-order structures such as rings, hourglasses, and gauzes. Septins are essential for (or play essential roles in) many cellular processes including cytokinesis, cellular morphogenesis, ciliogenesis and spermatogenesis, and fungal

pathogenicity. In the budding yeast *Saccharomyces cerevisiae*, the septins localize to the bud neck and function as a scaffold for proteins involved in cytokinesis and other processes and as a membrane diffusion barrier preventing the intermixing of membrane-associated proteins between mother and daughter compartments. Based on their importance and ability to adopt a variety of architectures, it is of no surprise that the septins are tightly regulated throughout the cell cycle. One such regulation is through phosphorylation by kinases such as Elm1 (an LKB1/PAR4-related kinase). Elm1 localizes with septins at the bud neck, regulates the morphogenesis checkpoint controlling the apical-to-isotropic switch in bud growth, and plays an ill-defined role in maintaining septin structure at the bud neck. To elucidate the mechanism underlying the last function, we imaged *elm1Δ* cells by 3D time-lapse microscopy, and found that the daughter-half of the septin hourglass was selectively lost shortly after bud emergence in this mutant. As a consequence, the morphogenetic checkpoint regulators at the daughter side of the bud neck were also lost, which could explain the elongated morphology associated with a loss of Elm1 function. Using platinum-replica electron microscopy to see the septin architecture at the filament level, we believe the mechanism for this instability of the septin hourglass may be through the inability of paired septin filaments to form efficiently in *elm1Δ* cells. Thus, the major function of Elm1 is to control the stability of the septin hourglass, specifically the daughter half. Cells lacking Elm1 also exhibit mild defects in cytokinesis. Strikingly, cells lacking both Elm1 and the septin Shs1 display major defects in cytokinesis, including actomyosin ring destabilization and defects in primary septum formation. These data suggest that Elm1 and Shs1 function in parallel pathways to regulate the septin architecture required for proper cell morphogenesis and cytokinesis in budding yeast.

P1528/B662

Dchs1 and the Septin Cytoskeleton: a Molecular and Developmental Etiology Underlying Mitral Valve Prolapse.

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Mitral valve prolapse (MVP) is a potentially life threatening cardiac disease that affects 1 in 40 individuals and is the most common reason for mitral valve surgery. MVP is defined by a disease of the valve leaflets which exhibit progressive disruption of the extracellular matrix (ECM) and is characterized by collagen fragmentation and increased proteoglycan and elastin production. As a result, the mitral valves enlarge and become biomechanically incompetent, resulting in leaflet billowing and failure to close during ventricular systole. There are no effective nonsurgical treatments for MVP and therapeutic efforts have been hindered by an incomplete understanding of its fundamental causes. Our group was the first to identify a genetic cause for MVP through identification of mutations in the atypical cadherin gene, *DCHS1, in* multiple families of non-syndromic MVP. In an effort to further define the intracellular mechanisms regulated by DCHS1, we recently performed a series of two-hybrid studies and co-immunoprecipitations (Co-IP's) and identified a novel protein complex consisting of DCHS1, Lix-1 like (LIX1L), and septin-9 (SEPT9). We performed a series of in *vivo* epistasis experiments that support the genetic interactions between *Lix1L and Dchs1* as we saw a synergistic effect of compound heterozygosity on mitral valve morphogenesis. We were able to refine the interaction between LIX1L and SEPT9 to a 36 a.a. binding domain, which allowed us to generate a cell permeant 36-mer peptide to target the distinct LIX1L-SEPT9 interaction in the following approaches. Since SEPT9 is able to crosslink and bundle actin monomers, we measured actin filament content with G-actin/F-actin polymerization

assays and immunocytochemistry analyses. We observed a significant decrease in septin-actin organization and defects in cell and nuclei shape in cardiac fibroblasts (CFs) deficient of *Dchs1, Lix1L,* or both. We also found that the DCHS1-LIX1L-SEPT9 (DLS) complex promotes cell, ECM, and actin filament alignment through application of a novel 3D *ex vivo* culture system that. These data support a mechanism in which DCHS1 based cell-cell adhesions regulate actin stability through LIX1L-SEPT9 interactions during critical stages of valve development.

P1529/B663

Reconstituting Cytoskeletal Assembly from Budding Yeast Extracts Reveals Basic Biophysical Properties of Septin Filament Polymerization.

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Cells build large, micron-scaled structures from relatively small, nanometer-scaled proteins. The septin cytoskeleton is particularly adaptable in its ability to form micron-scale structures in a variety of shapes and sizes for various functions. Septins are GTPase proteins that self-assemble into oligomeric “rod-shaped” subunits 17-32 nm long, and are conserved from fungi to mammals. These oligomers can then polymerize into much larger filaments at the plasma membrane or in association with other cytoskeletal proteins. Filaments can be arranged into higher-order assemblies such as laterally associated bundles, crosslinked lattices, wrapped gauzes, and curved rings. Higher-order assemblies serve as platforms for protein localization including the polymerization of other cytoskeleton proteins, scaffolds for cytokinesis and cell signaling events during morphogenesis, and act as protein diffusion barriers. Septin assemblies go through substantial rearrangements throughout the cell cycle suggesting that septin filament polymerization is finely tuned and regulated. Much about what is known about actin and microtubule assembly came from seminal studies that uncovered biophysical properties underlying polymerization. A network of regulatory proteins modulate actin and microtubule biophysical properties to build and arrange higher-order assemblies. Very little is known about how cells control where and when septin filaments polymerize, and how filaments are arranged into higher-order assemblies. Genetic studies in yeast have identified a suite of potential septin regulators, including septin-associated kinases, cell polarity proteins, and cell cycle regulators. However, how these regulators mechanistically modulate septin filament polymerization or filament arrangements remains mysterious. To uncover the biophysical properties of septins and to investigate how regulators tune polymerization, we married a filament polymerization reconstitution system with cell extracts from budding yeast. Extracts are incubated on supported phospholipid bilayers and individual filaments are imaged by TIRF microscopy and SEM. We found that septin filament polymerization is most consistent with it being an isodesmic rather than cooperative process. We also found septins from extracts polymerize into distinct spatial patterns different from filaments polymerized from recombinant protein. Moreover, SEM has revealed a new, undescribed filament arrangement found from extracts. This combined extract-reconstitution approach enables us to dissect the underlying biophysical mechanisms of septin polymerization while simultaneously affording control of its regulators by utilizing the power of yeast genetics.

P1530/B664

The Role of Atx-2 and Vpr-1 on Sperm Positioning in the *C. Elegans* Meiotic Embryo.

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The role of ATX-2 and VPR-1 on sperm positioning in the *C. elegans* meiotic embryo Fertilization occurs during female meiosis in most animals, which raises the question of what prevents the sperm body (DNA, centrioles, and organelles) from interacting with the meiotic spindle. In a previous study, we found that *Caenorhabditis elegans* sperm DNA stays in a fixed position at the opposite end of the embryo from the meiotic spindle while yolk granules are transported throughout the embryo by kinesin-1. In the absence of F-actin, the sperm body was transported with the yolk granules, resulting in sperm DNA within 2 µm of the meiotic spindle. We are currently exploring the possibility that contacts between the maternal ER, paternal mitochondria, and cortical F-actin may play a role in tethering the sperm body to the posterior end of the embryo. In preliminary studies we found that depletion of Ataxin-2 (ATX-2) caused disruption of the sperm body, the incorrect positioning of sperm DNA, and the scattering of paternal mitochondria. In contrast, the sperm body remained intact upon depletion of VAP-B (VPR-1), but was incorrectly positioned within the embryo. Further studies are needed to deduce how these proteins are specifically involved in correct sperm positioning inside the embryo.

P1531/B665

Climp-63 Regulates Nuclear Movement during Polarization of Migrating Cells.

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In our lab we are interested in understanding how cells position their nuclei in different biological contexts and the role for nuclear positioning. The connection between the cytoskeleton and the nucleuskeleton are crucial for most of these events, however how the endoplasmic reticulum (ER) is organized during cellular polarization remains to be elucidated. The ER is a dynamic organelle with three functional and morphological domains, the nuclear envelope, flat sheets and reticular tubules. Being the largest cell organelle, its morphology is also the most complex. Using migrating NIH 3T3 fibroblasts in a wounding assay where nuclei are repositioned away from the leading edge, we found that ER sheets accumulate at the perinuclear region, while ER tubules distribute along the leading edge upon nuclear movement. Moreover, by performing FIB-SEM we observed that nuclear positioning was correlated with bigger and more continuous ER sheets at the perinuclear region. Therefore we tested if ER morphology could regulate nuclear positioning in the wound assay. To this end, we depleted different ER proteins previously reported to be involved in ER morphology and found that depletion of Climp-63, a sheet-enriched ER transmembrane protein thought to be responsible for ER luminal spacing, prevents nuclear movement away from the leading edge. In addition, cells without Climp-63 failed to increase perinuclear ER accumulation during nuclear movement. Nuclear movement away from the leading edge is mediated by actin retrograde flow and actin dorsal cables attached to the dorsal side of the nucleus via TAN lines. We tested if depletion of Climp-63 had any effect on actin cytoskeleton, either on dorsal or ventral actin, and on TAN lines. We found that the speed of actin retrograde flow and the number of TAN lines upon Climp-63 depletion were not different from the control. Surprisingly, we found that the number of ventral actin stress fibers under the nucleus increased. In the ventral side of the nucleus we observed that ER was wrapped around ventral actin cables. In the absence of Climp-63, the ER area and thickness in the ventral side of the nucleus was decreased and we observed less wrapping of the ER around the

actin cables. Given these results, we propose that ER membrane might be necessary to wrap around actin cables under the nucleus to prevent the connection of ventral static actin cables to the nuclear envelope proteins and allow the nucleus to slide on top of immobile ventral actin cables.

P1532/B666

Netosis Proceeds by Cytoskeleton and Endomembrane Disassembly and Pad4-mediated Chromatin De-condensation and Nuclear Envelope Rupture.

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Neutrophil extracellular traps (NETs) are web-like DNA structures decorated with histones, antimicrobial and cytotoxic proteins released by activated neutrophils. Initially described as a way for neutrophils to trap and neutralize pathogens during innate immunity, NETs are now widely implicated in the detrimental effects of several autoimmune diseases. Peptidylarginine deiminase 4 (PAD4) citrullinates histones and is required for NETs formation (NETosis) in mouse neutrophils. While various molecular mechanisms mediating NETosis are being revealed, the cellular events driving NETs release are still unclear. Here, we determined the sequence of cellular events in NETosis, and examined the role of PAD4 in these events. We performed high resolution time-lapse microscopy of mouse and human neutrophils (PMN) and differentiated HL60 neutrophil-like cells (dHL-60) labelled with fluorescent markers of the cytoskeleton and organelles and stimulated with ionomycin or lipopolysaccharides to induce NETosis. We found that stimulated neutrophils eject NETs after decondensation of the nuclear DNA in the nucleus, rupture of the lamin meshwork and nuclear membrane allowing the release of decondensed DNA to the cytosol, disassembly of the actin, microtubule and vimentin intermediate filaments networks, vesiculation of the endoplasmic reticulum and plasma membrane and finally rupture of the plasma membrane. More importantly, we found that these cellular pathways occur in a specific and well conserved temporal order suggesting a requirement for a precise sequence for progression through NETosis. Indeed, inhibition of actin disassembly, one of the first cellular events, blocked NETs release. To examine the role of PAD4 in NETosis we isolated neutrophils from PAD4-deficient mice and generated a PAD4-knock down (KD) HL60 CRISPR line. We found that chromatin de-condensation, lamin meshwork and NE rupture and extracellular DNA release required the enzymatic and nuclear localization activities of PAD4. Thus, NETosis proceeds by a well conserved, step-wise sequence of cellular events culminating in the PAD4-mediated expulsion of DNA. Our data further suggest that targeting these cellular pathways might be a better approach for controlling NETosis progression than targeting the various divergent signaling pathways. Indeed, plasma membrane microvesicle shedding, actin and lamin meshwork disassembly, DNA de-condensation, nuclear envelope and plasma membrane rupture are now potential therapeutic targets for NETosis inhibition.

P1533/B667

Curvature Sensitive Septin-membrane Interaction Drives Membrane Deformation.

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Septins are cytoskeletal proteins that assemble into a variety of supramolecular organizations from paired filaments to bundles, ring like structures or gauzes of orthogonal filaments [1]. Septins are bound to the inner plasma membrane through specific interactions with phosphoinositides [3]. Septins are essential for cytokinesis, participate in the formation of diffusion barrier and might be involved in membrane deformation and rigidity. Septins are localized in situ at sites displaying a micrometric Gaussian curvature. We have used a combination of in vitro bottom approaches and theory to analyze how the curvature sensitivity of septins could induce membrane deformations. Besides we analyzed in details the septin-membrane interaction. We have shown that septins arrange differently on positive or negative curvatures [4] using scanning Electron Microscopy imaging on undulated PDMS substrates. Besides, this curvature preference is closely related to the ability of septins to reshape and deform membranes. Indeed, bound to Giant unilamellar Vesicles (GUVs), septins induce striking deformations with regular spikes and hollow micrometric deformations at the surface of liposomes, as visualized by fluorescence microscopy. Smaller vesicles (LUVs of 100-300 nm in diameter), highly positively curved, are flattened by Septin filaments into “pancake” like objects as shown in 3D by cryo-electron tomography. With the resolution of cryo-EM we visualize both the septin filaments and the deformed vesicles. To get more details into the Septin-membrane interaction at the molecular scale we have started carrying out sub-tomogram averaging. Preliminary analyses highlight the orientation of the Septin domains interacting with the membrane. **References**: [1] A. Bertin, et al. (2010), Phosphatydinositol 4,5 biphosphate promotes budding yeast septin filament assembly and organization, J. Mol. Biol., 404(4), 711-31. [3] A.Bertin, et al. (2008), Saccharomyces Cerevisiae septins: supramolecular organization of heterooligomers and the mechanism of filament assembly, Proc.Natl. Acac. Sci USA, 105, 8274-8279 [4] A. Beber et al. (2019), Membrane reshaping by micrometric curvature sensitive septin filaments, Nat. Commun., 10, 420.

P1534/B668

The 3D Dynamics of Macropinosome Formation and PI3K Activity in Macrophages Revealed by Lattice Light-sheet Microscopy.

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Macrophages are sentinels of the innate immune system that are constantly sampling solutes within their environment. This immune surveillance is accomplished by creating actin-rich membrane ruffles that are shaped into macropinosomes filled with extracellular fluid in a process generally requiring phosphatidylinositol 3 kinase (PI3K) mediated activity. To correlate membrane reshaping with the timing and localization of PI3K activity, we used volumetric imaging of macrophages dually expressing fluorescent protein probes for the plasma membrane (Lck membrane targeting sequence) and for PI3K products (Akt PH domain). Macrophages exposed to different polarizing stimuli formed macropinosomes with diverse morphological mechanisms. In resting macrophages, the predominant form of macropinocytosis we observed fits the canonical model of linear ruffle extension, followed by

ruffle circularization, and cup closure mediated by fusion of the ruffle with the cell surface. By combining multiple volumetric representations, we observed that many macropinosomes also formed at the base of large sheets during the extension phase. Following a growth factor starvation and stimulation sequence, a large circular dorsal ruffle formed that restricted diffusion of PI3K products to the inside of the ruffle, and many macropinosomes were formed in a flurry of membrane activity as the ruffle constricted. Stimulation with lipopolysaccharides produced clusters of dorsal ruffles rich in PI3K products that moved around the surface and produced multiple macropinosomes. Using chemical inhibition of PI3K, we demonstrated the role of $PI(3,4)P_2/PIP_3$ in coordinating membrane circularization. Inhibition did not impact ruffling activity throughout the cell, but dysregulated cup closure and resulted in failed macropinosome formation. The improved spatiotemporal resolution by lattice light-sheet microscopy combined with advanced image analysis enabled a more quantitative view of the membrane dynamics and PI3K signaling controlling macropinosome formation.

Chemotaxis and Directed Cell Migration 1

P1535/B669

Coupling between Eukaryotic Cell Shape and Polarity: a Route to Micron-scale Curvature Sensing?

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A cell's crawling direction is in part controlled by the polarization of Rho GTPase activity on the cell. Spontaneously-developing polarization in Rho GTPase activity can be sensitive to the shape of the cell. Reaction-diffusion simulations of Rho GTPase dynamics show that regions of high activity localize to the narrow end of elongated cells. We earlier showed [Camley et al. Phys. Rev. E **95**, 012401 2017] that when Rho GTPase activity drives cell protrusion, and cell shape reorganizes Rho GTPase signaling, this positive feedback loop can create persistent turning trajectories of cells. Persistent turning very similar to our predictions has recently been observed experimentally in keratocytes [Allen et al. BioRxiv doi:10.1101/443408]. Can the coupling of Rho GTPase activity to cell shape provide a route for cells to use nanometer-sized proteins to sense their shape even when the membrane is curved on the micron scale? We extend these ideas to understand which membrane shape features control the localization of Rho GTPase activity. We show that we can develop an "energy landscape" for Rho GTPase activity as a simplified model that describes where Rho GTPase activity occurs as a function of the membrane shape; this landscape predicts the results of more complex reaction-diffusion simulations. We find that the accuracy of shape sensing depends on an interplay between the size of the area of high Rho GTPase activity, the shape of the membrane, and the roughness of the membrane around its average shape, with larger domains of activity being more robust to roughness.

P1536/B670

Leader Cells in Collective Chemotaxis: Optimality and Tradeoffs.

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Clusters of cells can work together in order to follow a signal gradient, chemotaxing even when single cells do not. Cells in different regions of collectively migrating neural crest streams show different gene expression profiles, suggesting that cells may specialize to leader and follower roles. We use a minimal mathematical model to understand when this specialization is advantageous. In our model, leader cells sense the gradient with an accuracy that depends on the kinetics of ligand-receptor binding while

follower cells follow the cluster's direction with a finite error. Intuitively, specialization into leaders and followers should be optimal when a few cells have more information than the rest of the cluster, such as in the presence of a sharp transition in chemoattractant concentration. We do find this - but also find that high levels of specialization can be optimal in the opposite limit of very shallow gradients. We also predict that the best location for leaders may not be at the front of the cluster. In following leaders, clusters may have to choose between speed and flexibility. Clusters with only a few leaders can take orders of magnitude more time to reorient than all-leader clusters.

P1537/B671

The Lamellipodium as a Migration Direction Decision Module.

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The lamellipodium is the 1-2 micron region at the front of the cell that evaluates the suitability of the surrounding extracellular matrix (ECM) for migration. Without the Arp2/3 network at the core of the lamellipodium, cells cannot detect ECM gradients. However, no physical link is known to connect the Arp2/3 network and integrin ECM receptors while the cell is probing and evaluating -- linkages form after integrins are bound to ECM and cells have accepted the ECM as suitable for migration. Here we show that despite not being physically bound to each other, the Arp2/3 actin network creates a high-density band of conformationally inactive, unligated integrins throughout the width of the lamellipodium. This high-density pool of integrin is dispersed when either the integrin tail is truncated or Arp2/3 is inhibited with CK666. Without the high density of unligated integrin in the lamellipodium, cells migrate slower and less persistently, changing direction more frequently than cells with intact lamellipodia. In contrast, alteration of the actin lamellipodium organization by inhibition of formins with SMIF2 does not displace the concentration of integrin, and SMIF2 treated cells migrate faster and more persistently than untreated cells. Neither treatment removes the very thin band of high-affinity integrins that outline the outermost edge of the lamellipodium. Together, these data suggest that the Arp2/3 actin network in the lamellipodium traps unligated integrin at the front of the cell to spatially evaluate the suitability of ECM - creating a migration direction decision module at the front of the cell.

P1539/B673

Collective Stabilization of Migratory Polarity Drives Cluster Dispersal in Vivo.

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Single cell migration programs shape tissue architecture during development. Often, such migrations initiate when subsets of cells separate from a cohesive group. Although much is known about how cells separate from an epithelium, the mechanisms underlying dispersal from dynamic, clustered cell ensembles remains poorly understood in *vivo*. Here, using the developmental migration program of Drosophila primordial germ cells (PGCs), we show that migratory polarity is stabilized to mechanically disrupt a motile cell cluster. PGCs utilize a G protein coupled receptor (GPCR), Tre1, to orient and maintain front-back migratory polarity radially from the cluster. Stabilization of the migratory axis positions myosin II dependent contractile forces at the rear to generate constant tension on cell-cell adhesions until rupture. Randomly migrating cells produce transient tension of equal magnitude on cell-cell adhesions but fail to separate, demonstrating a temporal tension threshold for detachment. We further show that migration based cluster dispersal is robust to increases in cell-cell adhesion and is more efficient when coordinated. Our results demonstrate that stabilization of the forces driving cell

movement are sufficient to disperse cell clusters under physiological settings and present a paradigm for how such events could occur in development and disease.

P1540/B674

Non-canonical Hh Signaling Directs Germ Cell Migration through Regulating Pi(4,5)p_2 and Actin Dynamics.

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Directional cell migration is a complex integrated process that requires coordinated changes in signal transduction, cytoskeletal structures and membrane organization. In tissue development and homeostasis, many cells acquire motility and migrate in response to guidance signals that direct cells to their destinations. Certain pathological conditions such as inflammation and cancer metastasis are directly related to abnormally migrating cells, emphasizing the importance of understanding the molecular and cellular mechanisms directing cell migration. Drosophila embryonic germ cell (GC) migration has served as an ideal model for studying directional cell migration due to the ease of tracking this cell population, the stereotypical migration patterns, and the wide range of cell biological and genetic tools available in Drosophila. Two distinct mechanisms have been proposed to guide GC migration. In the attractant model, Hedgehog (Hh) secreted by the somatic gonadal precursors (destination cells) attracts migrating GCs. In the repulsion model, a repellent signal generated by localized lipid phosphatases prevents GCs from straying from their correct migratory routes. Here, we report a set of findings that support a role for Hh signaling in GC migration through the GPCR Tre1 and its downstream effectors. First, we showed that GCs null for *Tre1* are fully capable of migrating but cannot navigate. Second, we showed that Tre1 induces local PI(4,5)P_2 synthesis by recruiting dPIP5K, a Drosophila PI4P 5-kinase, and that localized dPIP5K and PI(4,5)P_2 overlap F-actin-enriched protrusions in GCs. Third, we showed that WASP, an actin nucleation promoting factor activated by PI(4,5)P_2, is required for GC migration and localizes at the F-actin protrusions. Fourth, F-actin protrusions were diminished in *Tre1* mutant GCs, together with loss of local concentrations of dPIP5K and PI(4,5)P_2. Fifth, Smo activation by Hh induced increased localization of Tre1 on the plasma membrane and increased Tre1 binding to dPIP5K. Taken together, these results indicate that Hh signaling steers GC migration by regulating Tre1 localization. Tre1 interaction with dPIP5K activates local PI(4,5)P_2 synthesis and local actin polymerization, through WASP, to generate the F-actin protrusions that “pull” migrating GCs toward the somatic gonadal precursors, the source of the Hh ligand. Our findings suggest that non-canonical Hh signaling through the Tre1 GPCR regulates the cytoskeleton to facilitate developmental cell migration. These findings have potential clinical implications for Hh-related diseases, including cancer metastasis.

P1541/B675

Mitofusin 2 Regulates Neutrophil Adhesive Migration and the Actin Cytoskeleton.

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Mitochondrial membrane potential is required for neutrophil migration, although the mechanism remains unclear. Here, we report that mitochondrial outer membrane protein Mitofusin 2 (Mfn2) regulates neutrophil homeostasis in vivo. *Mfn2*-deficient neutrophils are released from the hematopoietic tissue and trapped in the vasculature in zebrafish embryos. Human neutrophil-like cells deficient with MFN2 fail to be arrested by activated endothelium under sheer stress or perform

chemotaxis on substrates. Deletion of Mfn2 results in a significant reduction of neutrophil infiltration to the inflamed peritoneal cavity in mice. *Mfn2*-deficient neutrophil-like cells and mouse embryonic fibroblasts display heightened Rac activation. Mechanistically, MFN2 maintains mitochondria-ER interaction and prevents excessive elevation of cytosolic calcium and subsequent phosphorylation of CaMKII upon stimulation. Inhibiting CaMKII or the Rac GEF Tiam rescues the chemotaxis defect that results from Mfn2 depletion. Altogether, we identified an Mfn2-CaMKII-Tiam-Rac axis in regulating neutrophil migration and discovered a role of *MFN2* in regulating the actin cytoskeleton.

P1542/B676

A Balance between Turning and Persistent Motion Is Critical for Fast and Efficient 3 Dimensional Neutrophil Migration.

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While much research has been dedicated to identifying the cascade of specific biochemical processes involved in the recruitment of neutrophils, much less is known about the mechanical events driving their directed migration. In particular, it is still unclear how neutrophils generate the necessary traction forces to migrate across three-dimensional (3-D) extravascular spaces and the effects of matrix porosity on turning events and persistence during this process. In this study, we examine the effects of extracellular matrix properties on the mechanics of 3-D neutrophil motility in collagen gels. We embedded neutrophil-like differentiated human promyelocytic leukemia (dHL-60) cells in collagen matrices of different concentrations containing fluorescent micro-beads. To induce directed migration, we introduced the chemokine formyl-Methionyl-Leucyl-Phenylalanine (fMLP) to dHL-60 cells in a custom build device. We then used brightfield microscopy to track chemotaxing cells and both confocal and fluorescent microscopy techniques to image the movement of the embedded micro-beads and fluorescently labeled cells. We find that turning events, mediated by the Arp 2/3 complex, are crucial for fast 3-D neutrophil migration. Additionally, the Arp 2/3 complex and myosin II play an increasingly important role in 3-D migration as matrices become denser. Finally, we find that cellular force patterns vary in response to matrices with various degrees of pore size heterogeneities and cell's intrinsic abilities to engage in path-finding and contractility. The results from our study show that neutrophils migrating in 3-D environments employ distinct mechanical mechanisms that depend on the local structure of their mechanical environments. Our work has implications for understanding how neutrophils interact physically with their surrounding environment what conditions may be necessary for fast 3-D neutrophil migration.

P1543/B677

Coordination of Actomyosin Contractility and Mitochondrial Positioning during Interstitial Neutrophil Migration in Live an esthetized an imal.

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Cell migration is a fundamental biological process in which membrane remodeling is a critical step occurring as a result of a constant re-arrangement of the actin cytoskeleton. Neutrophil migration has been particularly studied due to their role in the immune response, and because their immune function is directly associated to their ability to migrate. Neutrophil migration induces extensive plasma membrane remodeling through a combination of protrusion generated by actin polymerization at the leading edge of the cell and retraction generated by non-muscle myosin II a (NMIIA) contraction at the cell uropod. In addition, membrane remodeling is energetically unfavorable and has to be supported by an adjustment of the cellular metabolic activity.Most of our knowledge on membrane remodeling comes from *in-vitro* 2-D model systems and only very limited work has been performed in more complex models or *in-vivo*. To this end, here we use intravital subcellular microscopy coupled to a computational analysis in 3D to understand the underlying mechanisms of the coordination among plasma membrane remodeling, actomyosin cytoskeleton and cell metabolism during interstitial neutrophil migration in the mouse ear. In our model, migrating neutrophils exhibit a very active and dynamic membrane remodeling with a continuous formation of micron-scale membrane protrusions, which interact with the tissue microenvironment (i.e. extracellular matrix (ECM)). Differently from what has been previously described, we observe that NMIIA is not only present at the uropod of the cell but also at the leading edge and in large lateral protrusions. In these new locations, NMIIA is not actively retracting membrane, and its localization is correlated with the architecture of the ECM. Furthermore, NMIIA recruitment at the leading edge is RhoA/ROCK independent, possibly indicating a new role for NMIIA in a machinery involved in sensing the ECM and a new mode of recruitment of NMIIA in neutrophil.Finally, we observe that mitochondria constantly reposition throughout the cells at the sites of NMIIA assembly, most likely in order to supply the ATP required to sustain the contractile activity, and change their polarization status during the various phases of migration. In summary, we have highlighted a novel correlation between mitochondria function and the dynamic rearrangement of the actomyosin complex *in-vivo*.

P1544/B678

The Role of Cytosolic Phospholipase A_2 during Neutrophil Chemotaxis.

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Neutrophils are the most abundant immune cells in human blood and are the first line of defense against any pathogenic insult. They play a key role in mediating and resolving inflammation, which is often the root cause of various diseases including asthma, arthritis, and heart disease. Inflamed and damaged tissues release Damage and Pathogen Associated Molecular Patterns (DAMPs/PAMPs) that serve as primary chemoattractants to recruit nearby neutrophils. These activated neutrophils secrete the secondary chemoattractant Leukotriene B_4 (LTB_4), which has been shown to dramatically amplify the range of the primary inflammatory signals and the robust recruitment of neutrophils. Defects in the ability to produce, secrete, or detect LTB_4 results in greatly attenuated responses to injury or

inflammation. LTB_4 production is a highly regulated process that is initiated by the interaction of primary chemoattractants with their cognate G-Protein Coupled Receptor. Following stimulation, cytosolic phospholipase A_2 ($cPLA_2$) translocates to the nuclear envelope (NE) and releases arachidonic acid (AA), which is converted to LTB_4 by the sequential action of 5-Lipoxygenase, 5-Lipoxygenase Activating Protein and Leukotriene A_4 Hydrolase. $cPLA_2$ plays a critical role in LTB_4 production as disruptions in its activity lead to a decrease LTB_4 production and neutrophil migration. $cPLA_2$ belongs to group IV calcium dependent PLA_2 family that hydrolyze the fatty acid chains on the *sn-2* position of triglycerides. $cPLA_2$ has six isoforms: α, β, γ, δ, ε and ζ. These isoforms share only 30% sequence homology with no redundancy in activity known. Interestingly, the status of different $cPLA_2$ isoforms in neutrophils and whether these isoforms are involved in LTB_4 production is currently unknown. We found that neutrophils express $cPLA_2$ α, β, γ, ε and ζ. Our results also demonstrate the over expression of GFP-$cPLA_2$α does not affect migration of *neutrophil-like* cells. Visualization of GFP-$cPLA_2$α dynamics reveal that it redistributes to the NE following stimulation with primary chemoattractants. These results indicate that GFP-$cPLA_2$ isoform overexpression system represents a useful system to elucidate the intracellular dynamics of $cPLA_2$ isoforms and to determine their role during neutrophil migration.

P1545/B679

Polo-like Kinase 4 Inhibition Modulates the Migration of Neutrophil-like Cells.

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Polo-like kinase 4 inhibition modulates the migration of neutrophil-like cells Lucas C. Klemm[1,2], Laurel E. Hind[2], Ryan A. Denu[3, 4], Mark E. Burkard[4], an na Huttenlocher[2] [1]Molecular and Cellular Pharmacology Graduate Training Program, University of Wisconsin-Madison, Madison, WI [2]Department of Medical Microbiology and Immunology, University of Wisconsin-Madison, Madison, WI [3]Medical Scientist Training Program, University of Wisconsin-Madison, Madison, WI [4]Division of Hematology/Oncology, Department of Medicine, University of Wisconsin-Madison, Madison, WI Neutrophils are fast-moving cells of the innate immune system and are often the first responders to sites of injury or infection. They are adept at both sensing and integrating complex gradients of multiple signals to migrate in a directional manner. Chemoattractants signal through G protein-coupled receptors, which activate downstream small Rho GTPases that induce neutrophils to polarize. The polarization of neutrophils is dependent on the maintenance of a “front” and “back”. “Frontness” and “backness” are mediated by specific signaling pathways and are maintained via a combination of feedback loops and cytoskeletal components such as microtubules. Here, we addressed the role of centrosome in neutrophil migration using both inhibitors and genetic tools. Inhibition of PLK4 with Centrinone B promotes neutrophil directed migration and induces robust frontness signaling with the formation of dynamic F-actin waves at the leading edge of neutrophil-like PLB-985 cells. We also find that PLK4 inhibition with Centrinone B leads to centrosome depletion. Genetic depletion of SAS-6, a protein necessary for centrosome duplication, also results in improved chemotaxis and the formation of actin waves at the leading edge. We find that Centrinone B does not significantly increase the velocity of SAS-6 depleted cells. Taken together, these findings suggest that the centrosome is not necessary for neutrophil directed migration and may negatively regulate the actin cytoskeleton and cell motility.

P1546/B680

Aurora Kinase a Coordinates Collective Migration by Determining Font Polarized Leader Cells.

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PROBLEM- Metastasis mediated by epithelial cancer cells often invade the neighboring tissue in a collective manner. BACKGROUND-Collective migration is reliant on leader cells which provide directionality cues to the migrating sheet. Numerous studies identified that leader cells possess heterogeneous phenotypes compared the other cells. Previously, we found that the abundance of phosphorylated HMMR-T703, a substrate for Aurora kinase A, predicts breast cancer specific survival and relapse-free survival in patients with estrogen receptor (ER)-negative, triple negative phenotype, or basal-like subtype breast cancers. Aurora kinase a (AurA) is a mitotic kinase which is important for centrosome maturation, duplication, spindle pole assembly and chromosome segregation. AurA is also found to play a role in cell migration as well as metastasis. METHODS: Live cell imaging was used to track the formation of leader cells during collective migration of mammary cells as well as the integrity of migrating sheet in wound closure assay. We used MCF10A cell line which is engineered with fluorescent labelled-tubulin a1b. These cells enable us to monitor the spatial-temporal pattern of centrosomes during migration. The localization and abundance of AurA, K14, and Cyclin B1 were assessed by fluorescence microscopy. Overexpression and impairment of AurA activity were achieved by lenti-viral transduction and small molecular inhibition, respectively. RESULTS- Here, through multi-parameter imaging of wound closure assays, we find that leader cells were found to have front-rear polarity whereas non-leader cells possessed random polarization. Small molecule inhibition of Aurora kinase activity, which is specifically expressed in leader cells, impaired centrosome polarity in leader cells. The loss of leader cells led to the loss of integrity of migrating sheet retarding collective migration. Overexpression of AurA elevated the migratory potential in wound closure assay by augmenting front polarization and microtubule nucleation of centrosomes. CONCLUSION- AurA specifically expressed in leader cells is important to drive collective migration by coordinating the migrating sheet as a whole. AurA plays a role in establishing front-rear polarity in leader cells which is one heterogeneous phenotype compared to the other cells. Together, these data indicate that AurA is one potential therapeutic target to influence the collective migration of human breast carcinoma cells.

P1547/B681

Migrating Neutrophils Execute Front Protrusion and Rear Retraction Programs with Certainty Until Doubt Or Completion.

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Chemotaxing neutrophils continuously engage in directional decisions as they navigate complex environments. During interactions with obstacles, neutrophils often develop multiple competing fronts, raising the question of how the cell is able to select which front to maintain and which front(s) to abandon. To tackle this question, we challenged HL60 neutrophil-like cells with an oval-shaped obstacle positioned symmetrically in 6 x 3 um microfluidic channels. Cells respond to the mechanical challenge by

creating two equivalent competing fronts that encounter identical chemotactic gradients. The symmetry breaks when one front starts retracting allowing the dominant front to drive the cell around the obstacle. From time-lapse microscopy data, we extracted hundreds of image features that report on cell shape and distributions of cytoskeletal components. Through a supervised statistical learning approach, we identified a small subset of features that carry predictive power; using these we asked how far back in time could we accurately forecast the direction outcome. Among the examined features, cell shape and actin features appear to have the largest predictive power. Surprisingly, we could predict the cell's turning direction with accuracy greater than 70% only during the last third of the decision-making process (about 18 sec before the initiation of retraction). In this context, cell decision-making does not apparently require amplification of pre-existing asymmetries. Previous work (Yang*, Collins* & Meyer, 2016, *Nat. Cell Biol.* **18**: 121) showed that Cdc42, a Rho-family GTPase, is predictive of cell turning in chemotaxing neutrophil-like cells. To determine if Cdc42 activity gives improved prediction of cell decision-making, we combined our microfluidic assay with cells expressing an optogenetic G-protein coupled receptor and a spectrally compatible FRET biosensor for Cdc42 activity. This system enables light-stimulated neutrophil "chemotaxis" while measuring downstream GTPase activity in the same cells. Repetitive stimulation to one of the competing fronts is sufficient to bias the cell's direction with a 90% success rate. Interestingly, administering stimulation only during the early phase of competition did not bias the cell's stochastic choice. This suggests that inducing a transient asymmetry at an early stage is not sufficient to drive the system out of its steady state. We hypothesize that the two fronts are independently executing their protrusion programs and only late in the competition they start "raising doubt" and are amenable to re-programming. In addition, we found that once a cell has made a decision, the losing front enters a refractory period that requires complete retraction to the cell body before stimulation can encourage a new protrusion.

P1548/B682

Modulation of Transplantable Photoreceptor Replacement Cell Chemotactic Mechanisms in Retinal Biomimetic Ligand Gradients.

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Disease or trauma induced loss of rod and cone photoreceptors is a major cause of vision loss worldwide. To replace photoreceptors and restore vision, laboratories around the world are investigating photoreceptor replacement strategies using subretinal injection of photoreceptor precursor cells (PPCs) and retinal progenitor cells (RPCs) [1-2]. A major obstacle in the field is that transplanted photoreceptor replacement cells exhibit extremely limited migration into host retina following injection into the subretinal space [1-3]. In this study, we show that PPC receptor expression and retinal ligand interaction modeling can target cell signaling pathways facilitating migration. In addition, we demonstrate that using systems pharmacology, drugs can be targeted to upregulate PPC receptors enhancing photoreceptor replacement cell migration. Previously, we developed a database of retinal ligand and PPC receptor pairs involved in migration [1]. Ligand-receptor pairs significantly

expressed and predicted to facilitate chemotactic migration included SDF-1/CXCR4, VEGF/KDR and Osteopontin/ITGAV. In this work we modeled the ligand-receptor activated cytoplasmic and nuclear signaling for SDF-1/CXCR4, VEGF/KDR, and Osteopontin/ITGAV to identify downstream mechanisms facilitating PPC and RPC migration (Fig. 2). We then visualized these three target receptors on the surface of both PPCs and RPCs (Fig.2). Next, we entered our PPC receptor data into a systems pharmacologic database to identify FDA approved drugs to modulate expression of receptors correlated to migration (Fig.3). Viability was evaluated in RPCs treated with drugs for 3hr, 6hr and 12hr at a concentration of 10μM (Fig. 4). Ligand-receptor driven PPC and RPC chemotactic migration was quantified using microfluidic channels and steady-state ligand gradients of VEGF, SDF and Osteopontin (Fig. 5). The data reveal combined bioinformatics modeling and systems pharmacology can be used to modulate of transplantable photoreceptor replacement cell chemotactic machinery to enhance migration toward improved transplantation outcomes.

Gap junctions

P1549/B684

Stimulation of A_{2B} Adenosine Receptors Enhances Activity of Connexin26 Hemichannels in Epithelial Cells of the Human Respiratory Airway.

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Inflammatory conditions of the respiratory airway upregulate the release of adenosine and change the expression of connexins (Cx). As a component of the lung's innate immune defense, adenosine participates in the mucus clearance and has anti-inflammatory and tissue protective effects in acute inflammation. In chronic conditions however, adenosine and Cx channels may contribute to inflammation firing and tissue detrimental events. We used Calu-3 cells to analyze the interplay between adenosine signalling and Cx channels in epithelial cells of the respiratory airway. The gold nanoparticle mediated laser perforation (GNOME-LP) intercellular dye transfer technique and dye uptake experiments were used for a quantitative estimation of the gap junction intercellular communication (GJIC) and the activity of Cx hemichannels, respectively. After we found that the adenosine receptor agonist NECA reduced the GJIC and enhanced the Cx hemichannel activity, we analysed the expressed Cx isoforms and adenosine receptor subtypes using quantitative RT-PCR and western blotting. Pharmacological agents and specific siRNAs allowed to determine the specific contribution of the different adenosine receptor subtypes, the induced signalling pathways as well as the respective connexin isoforms. We found that the NECA-related increase of the dye uptake rate depended on increased cAMP synthesis and subsequent activation of the protein kinase a (PKA). This reaction was predominantly related to stimulation of the A_{2B} adenosine receptor subtype. Moreover, the NECA-related enhancement of the dye uptake was accompanied by an upregulation of Cx26 expression and protein synthesis. The findings suggest that in epithelial cells of the human respiratory airway, stimulation of A_{2B} adenosine receptors activates the cAMP/PKA pathway, resulting in upregulation of Cx26 expression and synthesis. Cx26 forms gap junction hemichannels in the cell membrane. The identified mechanism integrates adenosine signalling and gap junction hemichannel activity and gives evidence that adenosine signalling and Cx channels may act together to promote persistent inflammation, the hallmark of various pathological conditions such as cystic fibrosis, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, or asthma.

P1550/B685

Localization of Critical Endocytic Molecules Needed for Gap Junction Plaque Internalization and an nular Gap Junction Formation.

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Gap junction channels facilitate the communication of molecules between two neighboring cells. Gap junction-mediated communication plays a key role in regulating cell activity during cell development, wound healing, and many other cellular processes. Gap junction channels, which are clustered into gap junction plaques, are eliminated from the cell surface by an internalization process that results in the formation of annular gap junction vesicles. Alterations in the rate of gap junction plaque internalization have been indicated to have profound consequences on intercellular communication and play a pivotal role in disease development. While the components of this internalization process are still insufficiently understood, it has been demonstrated that internalization involves a clathrin-mediated gap junction membrane invagination, which results in a dynamin-dependent release of annular gap junction vesicles into the cytoplasm of one of two contacting cells. We hypothesize that for internalization to occur, endocytic molecules would need to align with the gap junction surface in only one of the two contacting cells. To test this hypothesis, the relative locations of the clathrin and its adaptor protein (AP-2) with gap junction plaques were visualized in contacting cells. Immunocytochemistry, transmission electron microscopy (TEM), and 3D computer-assisted reconstruction methods were used to study gap junction plaque internalization in a human adrenocortical tumor cell line that expresses connexin 43 gap junction protein. We demonstrated the preferential association of clathrin and its adaptor protein (AP-2) with the gap junction plaque face in one of the two adjacent cells. Specifically, the association of AP-2 with the surface of gap junction plaques in one cell (70 ± 10% SEM) was more prevalent than the association with both faces of the gap junction plaques in the two contacting cells (30 ± 8% SEM). These findings, along with the corresponding observations made with TEM in which most annular gap junction vesicles were devoid of material adjacent to their inner (luminal) membrane, are consistent with the preferential attraction of clathrin and AP-2 to the gap junction plaque membrane in one of the two neighboring cells. Further, we suggest that there is a repulsion of these endocytic molecules in the adjacent cell. The attraction of endocytic molecules to one face of the gap junction plaque and exclusion from the other face would thus facilitate gap junction plaque internalization. Understanding the mechanisms of gap junction plaque removal from the cell surface will expand our knowledge of gap junction protein trafficking, turnover, and cell-cell communication. Supported by NSF Grant #MCB-1408986

P1551/B686

Endocytosis Across the Gap Junction Connexin Protein Family.

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Proteins fated to be internalized by clathrin mediated endocytosis require an endocytic motif where AP-2 or other adaptors can bind and recruit clathrin. Tyrosine and di-leucine based sorting signals have been characterized to be such canonical motifs. Studies by us and others have shown that Cx43 has two canonical tyrosine based endocytic motifs that recruit clathrin and mediate its endocytosis. Here, we have examined amino acid sequences across the connexin protein family to identify canonical and possible non-canonical endocytic motifs to determine how non-Cx43 gap junctions might be

endocytosed. An alyses showed that surprisingly, quite a number of mammalian connexins do not contain any recognizable endocytic motifs. Moreover, through Clustal Omega sequence alignment, we have shown that unexpectedly, there is very little conservation of endocytic motifs between connexins, except for between Cx30 and Cx32. While most connexins that have recognizable endocytic motifs have them located in the C-terminal portion, some have canonical signals instead located in the intracellular loop (e.g. Cx36). We then demonstrate through an assortment of GFP-tagged connexins that either contain or do not contain recognizable endocytic motifs and video microscopy, that even endocytosis of connexins without recognizable endocytic motifs can occur; even in cells that do not express any other connexin type (HeLa, MDCK2 cells). Thus, the endocytic mechanisms that are embodied across the connexin protein family are unexpectedly diverse and needs to be investigated further. The reason for this diversity currently is not known, but it is tempting to speculate that it correlates with connexin-species function, and/or the tissue the connexin type is expressed in. An alyses towards this end are currently under way.

P1552/B687

Unveiling Disease Relevance in Gap Junction Turnover.

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Mutations in the gap junction (GJ) forming proteins, connexins (Cxs) are known to cause a diverse array of severe human diseases, including hearing loss, neuropathies, skin disorders, and cancer. Interestingly, the half-life of GJ channels is short (1-5 hours) suggesting that regulated turnover plays an important role in GJ function. Over the past years we have characterized in great detail the molecular machinery that turns over GJs, and we identified a number of specific amino acid residues in the Cx43 (the most widely expressed and best studied GJ protein) C-terminal tail that are critical regulators of GJ turnover. To test the hypothesis that mutations on these critical sites could function as a disease mechanism, we established the CRISPR/Cas9 gene-editing technology in the zebrafish model organism. We expect that our analyses will show whether mutations on turnover-relevant residues can lead to disease and to what phenotypes; an important unaddressed yet likely disease mechanism. As impaired GJ turnover should negatively affect cell migration that is particularly critical during development, we expected primarily developmental phenotypes (including embryonic lethality). Our hypothesis is supported by the fact that a large number of healthy humans (over 60,000 individuals represented in the ExAC data base) do not exhibit mutations of any of the residues that we have identified. We began by replacing wild type Cx43 with a mutant in which all of the identified amino acid residues critical for endocytosis were deleted (Cx43ΔCT). However, this mutant still expresses the critical residues that allow it to be trafficked to the plasma membrane and form gap junction channels. Surprisingly, Cx43ΔCT homozygous zebrafish develop to adulthood, but show a significant increase in their heart rate, abnormal hematopoiesis, and a significant lengthening of fin segments, indicating mis-regulated development in organs known to be affected by Cx43 mutation. In addition, a more specific Cx43 mutation (Cx43Δ256-289) which deletes only some of the critical residues involved in gap junction turnover shows different phenotypes, such as a decrease in heart rate and normal fin segment lengths. Currently, we are testing whether the surviving fish are rescued by another connexin type that takes over the functions of Cx43, e.g. Cx40.8. Cx40.8 is a close relative of Cx43 that is the relict of a gene duplication that occurred in pre-historic time in the teleost family. We are also testing the causes of these phenotypes by analyzing the molecular properties of these mutated gap junction channels and how they affect the function of the cells and tissues that rely on them.

P1553/B688

Complex Processing of Internalized Gap Junctions.

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Gap junctions connect cells of nearly all tissues in both vertebrates and invertebrates. While they are traditionally thought of as stable, long-lived structures, gap junctions are actually very dynamic in some tissues, turning over within a few hours after they form. The best-described mode of turnover is when one of the two connected cells engulfs the entire gap junction along with cytoplasm and membrane from the neighboring cell to form a double membrane vesicle, commonly referred to as a connexosome. The processing of connexosomes is poorly understood and is of interest in light of evidence that connexins have functions in addition to forming cell-cell channels. We are studying the ultrastructure of connexosomes within ovarian granulosa cells, which have a large number of these vesicles after cells are stimulated with luteinizing hormone. Using 3D electron microscopy with immunogold labeling, we have categorized and measured around 200 such internalized structures that contain Cx43 in a defined volume of tissue. We find that these structures have a wide variety of features. While slightly more than half fit the definition of a connexosome, many complex structures contain membrane regions that lack Cx43. Several structures contain intraluminal vesicles like those found in maturing endosomes, while a few contain whole organelles including mitochondria. An other subset appears to be undergoing lysosomal degradation. Notably, we have found similar double membrane vesicles in liver comprised in part of Cx32 gap junctions. Our ultrastructural study shows that by way of internalization, gap junctions can transfer bulk cargo to neighboring cells. The fascinating array of vesicles comprised of connexins shares structural similarities with both autophagosomes and endosomes. In essence, our studies provide insight as to how connexins and gap junctions could function beyond the plasma membrane.

P1554/B689

Phosphorylation Opens Up the C-terminal Domain Packing of Connexin43 Giving Enzymes and Endocytic Machinery Components Access to Aid in Gap Junction Endocytosis: Evidence by Molecular Modeling.

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Gap junctions have a surprisingly short half-live of only a few hours, an unusual and not well understood feature for a membrane channel that can be regulated by gating. The short half-live suggests that gap junction channels, soon after accrual to the plaque reach a stage where they no longer can fully function, and hence need to be endocytosed and replaced with functional channels. Using live-cell photobleaching, Dendra2-photoconversion, and successive FlAsH/ReAsH staining techniques combined with time-lapse imaging provided evidence for such a fast turnover, as well as accrual of newly synthesized gap junction channels predominantly along plaque peripheries and a simultaneous internalization of older channels from plaque centers. To better understand gap junction endocytosis, we have characterized in detail the molecular events that lead to gap junction internalization. We found that a series of post-translational modifications, ZO-1 binding and release, phosphorylation/de-phosphorylation on specific Cx43 C-terminal residues, as well as K63-poly-ubiquitination transitions functional into non-functional channels that then can interact with AP-2/clathrin and be endocytosed. We showed that ZO-1 - probably because of its huge size sterically hindering access of kinases, phosphatases, and E3-ubiquitin ligases to the Cx43-CT - needs to be removed from Cx43 before

endocytosis of gap junction channels can occur. We also showed that phosphorylation, on critical serine residues (e.g. S368, S279/S282), and ubiquitination needs to occur before AP-2/clathrin can bind and internalize Cx43-based gap junction channels. Based on Solan, Lampe, and Sorgen work showing that S365 de-phosphorylation/S368 phosphorylation causes a conformational alteration of the Cx43 C-terminal domain, we hypothesized that phosphorylation opens up the packing of the C-terminal domains to allow access of the endocytic machinery to the Cx43-CT, and to internalize gap junction channels. We built a hybrid-Cx model consisting of the low-energy solution structure of the Cx43-CT (published by the Sorgen lab) fused to the atomic resolution structure of Cx26 (published by the Tsukihara lab) and used CHARMM-GUI membrane builder software to model gap junction channels with un-phosphorylated and phosphorylated (S368, S279/S282) C-termini. Modeling demonstrates that phosphorylation indeed opens up the cytoplasmically located Cx43-C-terminal domains providing support for our hypothesis, and a molecular mechanism for how endocytosis of Cx43-based gap junction channels is regulated.

P1555/B690

Connexin43 (Cx43), Fin Regeneration and Bone Composition and Density in Zebrafish.

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Zebrafish have the remarkable ability to regenerate missing caudal fin tissue. This ability provides a model system to study the mechanisms responsible for the control of bone growth. During the growth process, new bony segments and joints are added to the distal tip of the caudal fin. This process is affected in several fin length mutants, including the *short fin* (*sof*) and *long fin* (*lof*) mutant. In the *sof* mutant, the number of segments produced is similar to wild type, however, the size of the segments is shorter; whereas, the *lof* mutant produces segments similar in size to wild type, but the number of segments produced is greater. The *sof* mutants develop short fins due to mutations in the gap junction gene *connexin43* (*cx43*). Mutations in human Cx43 can cause Oculodentodigital dysplasia (ODDD), a disorder characterized by craniofacial and skeletal malformations. Connexins are subunits of gap junctions which serve as intracellular passageways for the exchange of molecules between cells, including the exchange of signals between osteoblasts that may coordinate bone growth. Gap junctional intercellular communication (GJIC) between these cells may be responsible for regulating segment length. We have shown that short fin mutants have fins that are half the length of wild-type fins, reduced levels of *cx43* mRNA and a reduction in the amount of cell proliferation in the mesenchyme of regenerating cells. Using a novel technique of targeted gene knockdown of *cx43* in regenerating fins, we were able to recapitulate the segment length defect as well as the cell proliferation defect in the *sof* mutants. We hypothesize that the level of intercellular communication via gap junctions in the population of dividing cells in the regenerating fin may regulate bone growth by regulating the level of cell proliferation. Defects in Cx43 may also affect gap junction channel structure which may affect channel function and GJIC. Differences in channel structure were visualized using electron microscopy (EM), Energy-dispersive X-ray spectroscopy (EDS) and Quantitative backscatter imaging (qBEI). Chemical composition differences including calcium and bone density variations between newly formed bone post amputation and old bone was examined to better understand the self-repairing nature of fracture healing. Naturally, as humans age or enter microgravity conditions, overall bone density decreases, therefore understanding the role Cx43 plays in cell-cell communication in coordinating a response to re-grow missing tissue combined with bone composition analysis in zebrafish can help to better understand bone fracture healing in humans.

P1556/B691

Reciprocal Relationships between Gap Junction Nexus Morphology and Cell Physiology.

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Vertebrate gap junctions are formed by multimeric transmembrane connexin proteins. Connexins are required in all human organs. The 21 human connexin isoforms are expressed in cell-type specific combinations. Our current research is focused on gap junction isoforms Cx43 and Cx47 which are expressed in astrocytes and oligodendrocyte cells of the brain along with other organs. Genetic deletion of either connexin can lead to dysfunction of the human nervous system. We previously found that the gap junction supramolecular complex known as the Nexus can exist in strikingly divergent stable or fluid states. Nexus fluidity was altered by redox levels, carboxyl-terminus sequence, and importantly, by addition of a fluorescent protein tag. *New research reported here is aimed at testing how the gap junction Nexus controls subcellular morphology and physiology outside of the gap junction plaque.* We found that gap junction plaques profoundly alter localization of other proteins in the vicinity of the junctional membrane area. Transgene expression and mutation studies indicated that the effects of gap junctions on cell morphology are generated by two or more distinct mechanisms. We will also describe new fluorescent protein tagging strategies that allowed us to circumvent long-standing impediments to gap junction cell research and new image analysis methods we developed to quantify experimentally induced changes to Nexus morphology. Additionally, we report results of confocal microscopy and single-molecule localization microscopy that confirm and extend previous reports by other groups of spatially-differential posttranslational modifications to connexins within the gap junction Nexus and a spatial association of endoplasmic reticulum to gap junction plaques. Based on these and other findings, we hypothesize that Nexus fluidity alters intercellular calcium signaling. Variability in size and shape of gap junctions makes testing this hypothesis difficult- even in cell culture- so we turned to mesoscale computational modelling. We are currently testing if Nexus fluidity alters intercellular calcium signaling by using computational simulations in which gap junction channel number and mobility can be precisely controlled and experimentally modified. In *general, the results we present reveal new aspects of gap junction control over cell morphology and intercellular signaling.*

4

Structure and Function of the Extracellular Matrix

P1557/B692

Low Adhesive Scaffold Type I Collagen Prepared from Porcine Skin Regulates the Expression of Genes Relating to Osteocyte Differentiation.

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[Background] Collagen has biocompatibility and biodegradability with tissue or organ, therefore, collagen is the most promising material for tissue engineering. In general, the binding of rat marrow mesenchymal cells (rMMCs) to collagen scaffold is considered an effective to promote osteocyte differentiation. However, it is not clear why collagen is a suitable scaffold. At the ASCB&EMBO 2017

Congress, we showed that low adhesive scaffold type I collagen (LASCol) has marvellous ability to induce osteocyte differentiation in short term. In this study, we report the ability of LASCol scaffold by analyzing focused genes of osteogenesis. [Methods] Rat MMCs ($5x10^4$ cells/dish) were cultured on LASCol coated-dish with osteogenic basal medium. The alkaline phosphatase (ALP) activity of rMMCs cultured on the LASCol coated dish was observed. To evaluate osteogenic differentiation, mature RNA was isolated and reverse transcribed, subsequently the cDNA was analyzed with the real-time RT^2 Profiler™ PCR Array Rat Osteogenesis (QIAGEN). In addition, the up-regulated genes were further validated by qRT-PCR with specific primers. [Results] Rat MMCs on the LASCol coated-dish formed spheroid bodies and most of them showed ALP activity. However, on the other scaffolds only 60%-70% of cells had the ALP activity. Thus, the contact with LASCol might increase the ratio of osteocyte differentiation. Interestingly, after three days culture only a few genes of 84 genes showed increasing or decreasing. Though the genes such as *Col2a1*, *Bglap*, and *Phex* up-regulated, the threshold cycle of *Runx2* gene was similar. In particular, *Phex* might be an important gene because of the relative expression was high. Probably, LASCol would have the ability to induce osteocyte differentiation of rMMCs by the different gene expression. [Funding] This work was supported by the Adaptable and Seamless Technology Transfer Program through target-driven R&D (AS2715177U to K.M.). We thank Nitta Gelatin Inc. that provided the collagen materials.

P1558/B693

Tenascin-c Prolongs Inflammation and Edema by Inhibiting Lymphangiogenesis.

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<Introduction>Tenascin-C (TNC), an extracellular matrix protein, is well known to activate inflammation responses via activating inflammatory cells or promoting angiogenesis. The purpose of our study was to examine the relationships between TNC and lymphangiogenesis, because recent studies have showed lymphatic vessels are associated with the resolution of inflammation.<Methods>We used a mouse tail lymphedema model in vivo, and human dermal lymphatic endothelial cells (HDLEC) in vitro.<Results>TNC-knock out (KO) mouse revealed decreasing lymphedema compared to WT mice. Deletion of TNC promoted the growing of lymphatic vessels and reduced the accumulation of macrophages in inflammatory lesions. Furthermore, the number of lymphatic vessels were significantly increased in TNC-negative area, compared to TNC-positive area in WT mice. To assess the lymphatic function, we injected Evans blue dye (EB dye) intradermally at the tip of the tail and measured the concentration of EB dye in the drainage lymph node. The concentration was significantly higher in TNC-KO mouse, indicating the better flow of lymph fluid in TNC-KO. These results suggested lymphatic vessels were increased in TNC-KO; in other words, TNC may inhibit lymphangiogenesis. Therefore, we examined the direct interaction of TNC and lymphatic vessels in vitro using HDLEC. We found that TNC significantly decreased the number of HDLEC. In addition, TNC decreased the proliferative cells and increased the apoptotic cells. TNC also disturbed the tube formation ability of HDLEC. Thus, TNC has negative effects on HDLEC and, therefore, we next examine how TNC affects on HDLEC. We found that TNC increased the phosphorylation levels of p38 MAPK and ATF-2. In addition, phospho-ATF-2 positive nucleus were significantly increased after TNC addition. Inhibition of p38 MAPK completely suppressed the TNC-induced effects on HDLEC. Thus, p38 MAPK/ATF-2 is critical for TNC function. Furthermore, inhibition of TGF-β receptors completely suppressed the phosphorylation of p38 MAPK and TNC-induced effects on HDLEC. However, interestingly, SMAD inhibitor did not suppress the phosphorylation of p38 MAPK and TNC-induced effects on HDLEC, although TNC increased the phosphorylation levels of

SMAD2/3 in HDLEC. Therefore, we focused on the non-canonical TGF-β signaling. TNC increased the phosphorylation level of TAK1, known as one of the non-canonical pathway. Inhibition of TAK1 suppressed the phosphorylation of p38 MAPK and TNC-induced effects on HDLEC. Thus, non-canonical TGF-β signaling was associated with the effects of TNC on HDLEC.<Conclusion>TNC inhibits lymphangiogenesis and prolongs inflammation and edema. TNC suppresses proliferation and promotes apoptosis of HDLEC via TGF/TAK1/p38/ATF-2 signaling pathway.

P1559/B694

Matrix Fibulin-1 Association with Epidermal Growth Factor Receptor Inhibits Its Activation and Function in Lung Cancer.

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Epidermal Growth Factor Receptor (EGFR) is a known promoter of tumor progression and is overexpressed in lung cancer. Growth factor receptors (including EGFR) are known to interact with extracellular matrix (ECM) proteins, which regulate their activation and function. Fibulin-1 (FBLN1) is a major component of the ECM in lung tissue, and its levels are known to be downregulated in non-small cell lung cancer (NSCLC). To study the role Fbln1 could have in regulating EGFR signaling and function in lung cancer, we performed siRNA mediated knockdown of FBLN1 isoforms FBLN1C and FBLN1D in NSCLC cell line (Calu-1). This while not affecting net EGFR levels did significantly increase basal and EGF mediated EGFR activation. Overexpression of FBLN1C and FBLN1D accordingly is seen to inhibit EGFR activation as well in Calu-1 cells. Loss of FBLN1C/FBLN1D and resulting EGFR activation promotes migration of these cells in wound healing assays. This is confirmed by inhibition of EGFR using Erlotinib. Mechanistically, both FBLN1 isoforms interact with EGFR, though their association is not dependent on its activation. Notably, we discovered this association is detectable in cell-derived matrix (CDM) of lung cancer cells. Calu-1 cells plated on CDM's derived from FBLN1C/FBLN1D knockdown cells interestingly show a significant increase in EGF mediated EGFR activation. This promotes cell adhesion and spreading on CDM derived from knockdown cells, which is inhibited by Erlotinib. This also reflects in a distinct increase in the membrane localization of activated EGFR in spreading cells. Together, our findings show FBLN1 as part of the ECM can bind and regulate EGFR activation and function in NSCLC cells, highlighting the importance of tumor ECM microenvironment in influencing cellular behavior of EGFR dependent cancers.

P1560/B695

Sculpting of Tissues by Tension-suppressed Degradation.

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Development of tissue morphology generally depends on matrix and reflects a balance of synthesis and degradation of matrix proteins. Homogeneously tensed tissues and collagenous materials have suggested that collagen degradation is suppressed by strain, but tissues often sustain non-uniform strains that could in principle sculpt the mechanics and morphology of tissue. Here we study such processes in beating embryonic chick hearts and in isolated mice tail tendons. Normal beating hearts are subjected to ~5% peak strain in a spatiotemporally coordinated contractile wave, and the hearts maintain their collagen mass until the contractile strain is suppressed by inhibition of myosin-II.

Endogenous MMPs then degrade the collagens of contractility-inhibited embryonic chick hearts within ~30-60 minutes based on addition or not of MMP inhibitors and quantitation by calibrated mass spectrometry. In tendon fascicles, collagen fibrils primarily orient along the tension axis of the tissue but heterogeneous strains and gradients were induced using three-point, adherent bending, with patterned photo-bleaching of a fluorescent dye and collagen-binding peptide on fascicles used to measure strain. Microstructure of "cell-free" fascicles was simultaneously imaged using Second Harmonic Generation (SHG) signal while deformed fascicles were exposed to collagenases i.e. purified MMP-1 or Bacterial collagenase. Within physiological strain limits (i.e. ~5-8%), the decrease in fascicle degradation rate (relative to strain-free) was nearly independent of collagenase type despite different cleavage mechanisms, and tissue locations sustaining higher strains showed the degradation rate became almost independent of strain magnitudes. Sequestration of collagen's cleavage sites by strain is likely because permeation and mobility of fluorescent collagenase and dextran are strain-independent up to ~5-8% strains. Both tissue systems under heterogeneous strains indicate a degradative loss and sculpting of tissue locations that sustain the lowest strains.

P1561/B696

Type I Collagen Expression After Thermal Injury in Larval Zebrafish.

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Burn injuries are a prominent clinical issue often complicated by systemic inflammation and risk of infection. However, few models have been developed to address the molecular mechanisms that regulate burn injury healing. We have recently developed a zebrafish burn injury model to study burn injury healing, and have shown that collagen fibers are undetectable by second harmonic generation imaging (SHG) rapidly after thermal injury. Many experimental therapies have been designed to repair the extracellular matrix including applying collagen-based matrices over the healing wound. However, the regulation of endogenous collagen reorganization or repair is not well understood. Here, we have identified that different populations of cells are activating expression of type I collagen after either burn injury or fin amputation. We have expressed fluorescent reporters under control of putative promoters of type-I collagen subunits: *col1a1a, col1a1b and col1a2*. Larval zebrafish are either burned using an electric cauterizer or the caudal fin is transected with a surgical blade. Fish are then longitudinally followed during repair, and activation of type I collagen is assessed by fluorescent protein expression. In unwounded developing tails, *col1a1a and col1a1b* are expressed by elongated cells on the interior of the fin. In addition, *col1a1a* is expressed in the notochord and nervous tissue in the developing fin. Expression of *col1a2* is primarily in epithelial cells. Following both burn injury and tail transection, *col1a1a and col1a1b* are expressed in expanding cell populations on the interior of the regenerating fin. In contrast, *col1a2* is expressed in epithelial cells along the wound edge, and in cells surrounding collagen projections that have been previously identified during tail regeneration. Overall, here we present a new model in which to study the regulation of collagen regeneration during burn wound healing. Further studies are exploring the signaling that regulates collagen expression and timing during burn injury regeneration.

P1562/B697

Regulation of Extracellular Matrix Gene Expression by Desmosomal Cadherins.

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Desmosomes are protein complexes crucial for maintaining cell-cell adhesion and integrity of tissues. These complexes are made up of proteins from three families: transmembrane cadherins (Desmoglein and Desmocollin) link adjacent cells in the extracellular space, armadillo proteins (Plakophilin and Plakoglobin) stabilize the intracellular plaque, and the cytolinker Desmoplakin (DP) connects the plaque to the intermediate filament network. Desmosomal proteins have also been shown to coordinate gene expression pathways required for processes such as proliferation, differentiation and cell migration. In particular, several lines of evidence have linked desmosomal proteins to gene expression of extracellular matrix (ECM) proteins. Loss of Plakophilin-2 or Desmoplakin causes increases in expression of fibronectin and collagen, while in contrast, loss of plakoglobin results in a significant decrease in expression of fibronectin. These data indicate that individual components of the desmosomal complex control ECM gene expression via distinct cellular signaling networks. In our study, we sought to investigate the role of desmosomal cadherins in ECM gene expression, via use of A431 cells lacking either Desmoglein-2 or Desmocollin-2, generated via CRISPR-mediated knock-out (Dsg2 KO and Dsc2 KO, kindly provided by Dr. Daniel Conway). Compared to control cells, Dsg2 KO cells demonstrated a dramatic (~10-fold) increase in expression of Fibronectin (Fn1), and relatively minor changes in expression of Collagen 1 (Col1a1) and Collagen 2 (Col2a1). In contrast, no major changes in expression of Fn1 was observed in Dsc2 KO cells. We show that loss of Dsg2 KO (but not Dsc2 KO) also caused a significant increase in expression of the pro-fibrotic signaling molecule transforming growth factor beta 2 (Tgfb2), but not Tgfb1 or Tgfb3. Increased expression of Fn1, Col1a1, Col2a1 and Tgfb2 was also observed upon siRNA-mediated knockdown of Dsg2 in A431 cells, verifying that these changes are not clone-specific or due to off-target CRISPR effects. While expression of other desmosomal proteins is unchanged in Dsg2 KO cells (mRNA and protein levels), the integrity of the junctional complex was expectedly perturbed, as observed via a significant increase in Triton-solubility of Plakophilin-2, Plakophilin-3, Plakoglobin and Desmoplakin. Nevertheless, siRNA-mediated knockdown of these proteins could not rescue Fn1 or Tgfb2 increases in Dsg2 KO cells, indicating that these changes are not due to mis-localization of other desmosomal components. Taken together, our study highlights a novel role for Dsg2 in mediating ECM gene expression, adding significant insight into the mechanisms by which desmosomal cadherins control the adhesive behavior of cancer cells.

P1563/B698

Identification and Structure Activity Relationship Study of an α-Dystroglycan Binding Peptide from the Laminin α2 Chain LG4-5 Modules.

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Laminin is a major component of the basement membrane and has various biological activities. Laminin consists of α, β, and γ chains, in which 5α (α1-α5), 3β (β1-β3), and 3γ (γ1-γ3) chains have been identified. The laminin α2 chains are specifically expressed the in skeletal muscle, peripheral nerves, brain, and capillaries. The laminin α2 chain interacts with integrin α3β1, integrin α7β1, syndecan, and α-dystroglyan (α-DG). Specific binding of the laminin α2 chain with α-DG is critical to maintain the

structure and function of skeletal muscle, and genetic disruption of the laminin α2 chain causes muscular dystrophy. The laminin α2 chain binds to α-DG through its C-terminal globular module of LG (laminin globular) 4-5 modules. Previously, we developed an assay system to identify α-DG binding peptides using an Fc-tagged recombinant α-DG and peptide-chitosan matrices. We screened α-DG binding sequences of the laminin α2 chain LG4-5 modules using 42 peptides covering the entire sequence of the modules. The peptides were synthesized with a CGG residue on their N-terminus and conjugated on maleimidebenzoyloxy-chitosan to construct a peptide-chitosan matrix. Four peptide-chitosan matrices (A2G77-, A2G78-, A2G80-, and A2G95-chitosan matrices) demonstrated the Fc-DG binding. In this study, we focus on the A2G80 peptide (VQLRNGFPYFSY) and have performed a structure activity relationship study to obtain stronger binding peptides to α-DG. We synthesized a set of alanine-substituted peptides and evaluated α-DG binding activity. Initial results suggested that the side chain of three amino acid residues (Asn, Pro, Ser) does not affect α-DG binding activity of A2G80. Based on these results, we synthesized an additional 36 peptides focusing on two amino acid residues (Asn, Ser) and reevaluated α-DG binding activity. Some peptides significantly enhanced the activity compared with that of A2G80, suggesting that these substituted residues are critical for the α-DG binding. These results are useful for understanding α-DG binding mechanism of A2G80 and these peptides have a potential to be used as a drug delivery tool that are selective for the skeletal muscle.

P1564/B699

Force-dependent Extracellular Matrix Remodeling Results in Altered Exosome Diffusion by Cancer Cells and Activates Stromal Fibroblasts.

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It is known cancer cells secrete cytokines inducing normal fibroblasts (NFs) to become carcinoma-associated fibroblasts (CAFs). However, it is not clear how the CAF-promoting cytokines can effectively navigate the dense ECM, a diffusion barrier, in the tumor microenvironment to reach NFs during the early stages of cancer development. In this study, we devised an organoid system to investigate the possible mechanism of CAF induction at early stages of breast cancer. We found that in a force-dependent manner, ECM fibrils are radially aligned relative to the tumor spheroid. The fibril alignment enhances the diffusion of exosomes containing CAF-promoting cytokines towards NFs. Suppression of force generation or ECM remodeling abolishes the enhancement of exosome diffusion and the subsequent CAF induction.

P1565/B700

Nanoarchitecture and Size-exclusion Barrier Function of the Enteric Glycocalyx Examined by Freeze-etching Electron Tomography and Macromolecular Diffusion Assay.

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The intestinal epithelium is the largest interface between our bodies and the external environment and functions as a selective barrier for both endo- and exogenous materials, including pathogens. Directly adhering to the apical surface of the epithelium is an extensive layer of glycoprotein network—the glycocalyx—which has been hypothesized to function as a protective diffusion barrier to the underlying

epithelium. Visualization of the enteric glycocalyx by conventional transmission electron microscopy has revealed the presence of a layer of dense filamentous proteins situated atop the brush border that appear as a fuzzy coat. This appearance is due to inadequate preservation of glycocalyx native structure. In order to obtain an in-depth understanding of glycocalyx structure-function relationship, we performed freeze-etching electron tomography to visualize the enteric glycocalyx architecture of the rodent intestine in its native hydrated state in combination with Dextran-based diffusion experiment to probe its diffusion barrier property. We observed micrometer-long filamentous proteins emerging exclusively from the tips of microvilli. These filaments displayed extensive lateral contacts resulting in a dense three-dimensional columnar network with a 30 nm mesh. Strikingly, filament termini converge to form globular structures that are ~30 nm apart with liquid-crystalline packing organized into a plane. We then tested the functional relevance of these ultrastructurally derived dimensions by incubating rodent small intestine with different molecular weight fluorescent Dextran. We observed that 3 kDa Dextran with small Stokes radius at approximately 1.2 nm readily diffuses across the glycocalyx layer. On the other hand, the diffusion of 2000 kDa Dextran with an estimated Stokes radius at 27 nm was impeded by the surface coat. Our findings show that the enteric glycocalyx forms a continuous complex columnar network that traverses throughout the intestinal tract and physically segregates the apical cell surface from the lumen. In addition, inter-filament mesh size and filament termini spacing form the basis of a size-exclusion filter that allows passage of vital nutrients while preventing micrometer-scale bacteria access to the apical surface. Taken together, we have established a renewed structural understanding of the enteric glycocalyx and provide a foundation for future explorations on how the architecture of this surface coat correlates with intestinal physiology.

P1566/B701

Identification of Biologically Active Site in Mouse Laminin Alpha3b Chain N Terminal Region.

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Laminin α3 chain is a kind of laminin that is one of the components of extracellular matrix, and is highly expressed in the skin to promote skin regeneration and wound healing. Two types of isoforms with different N-terminal domains, α3A chain and α3B chain, have been identified for laminin α3 chain. Both laminin α3A chain and α3B chain are derived from the LAMA3 gene, and the α3B chain is a huge protein consisting of 1,800 amino acid residues with multiple globular domains at the N-terminus. On the other hand, the α3A chain has a structure in which most of the globular domain of the α3B chain is deleted. In this study, synthetic peptides covering the amino acid sequence of the N-terminal region of laminin α3B chain were prepared, and the biological activity of the peptides was evaluated for the purpose of elucidating the function of the N-terminal region of laminin α3B chain. A peptide consisting of 12 residues in length covering the amino acid sequence of the mouse laminin α3 chain N-terminal region was designed, and 188 peptides were synthesized. Human dermal fibroblasts (HDF) were used to evaluate the cell adhesion activity, and EDTA/heparin was added to the peptides that showed adhesion activity to identify the adhesion mode. In addition, neurite outgrowth activity was evaluated using rad adrenal pheoxhromocytoma cells (PC12). Twenty-one peptides showed adhesion activity to HDF, and 6 peptides showed neurite outgrowth activity. Of the 21 types that showed adhesive activity, 5 were heparin only, 3 were EDTA only, and 8 were both heparin and EDTA, and cell adhesion activity was inhibited. In addition, most of the active peptides were located in the globular domain of the laminin α3B chain. Many active peptides have been found in the globular domain at the C-terminal of laminin α3

chain. Similarly to laminin α3B chain, active sites are reported to be concentrated in the globular domain in laminin α1, α2, and α5 chains with N-terminal region, and they also play important functions in actual proteins. It has been suggested that the results of this study may provide useful information for elucidating the biological activity of laminin α3B chain.

Integrins and Cell-ECM Interactions

P1567/B702

The Pix Pathway Directs Assembly of Integrin Adhesion Complexes in Muscle.

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Integrin adhesion complexes (IACs) are called focal adhesions in non-muscle cells, and costameres in muscle cells. These sites are crucial for the adhesion of cells to ECM and are required for transmission of lateral forces between muscle cells. The composition of IACs is well established, however the mechanism that determines when and where IACs will form is unknown. *C. elegans* striated muscle has IACs at 3 locations: M-line, dense body, and attachment plaque at muscle cell boundaries. From a screen for mutants that disrupt the localization of IAC component PAT-6 (α-parvin), we discovered that loss of function of *pix-1* results in the absence of PAT-6 at muscle cell boundaries, but not at the M-lines and dense bodies. PIX-1 is the nematode ortholog of human β-PIX. Muscle-specific expression of wildtype PIX-1 cDNA rescues the muscle cell boundary phenotype. *pix-1* mutants lack additional IAC components at muscle cell boundaries and display reduced locomotion, yet sarcomere structure is normal. PIX-1 localizes to all 3 IAC structures in muscle. Mutations in genes encoding proteins at all known steps of the PIX signaling pathway show defects at muscle cell boundaries. Mutation of the highly conserved P190 residue in RhoGEF domain of PIX-1 causes defective boundary structures but yields normal levels of PIX-1 protein. A homology model of the RhoGEF domain of PIX-1, based on the known structure of human β-PIX, together with molecular dynamics simulations, indicates that the P190S mutation disrupts the structure of the RhoGEF domain, and enhances its interaction of GTP-bound (active) Rac, thus perhaps reducing the rate of the GTPase cycle. Rho GTPases function as molecular switches between active and inactive states, facilitated by GEF and GAP proteins, respectively. However, a GAP for PIX-1 in nematodes or β-PIX in mammals has not yet been identified. Based on previous studies it is hypothesized that the terminal phenotype of loss of function for a GEF and a GAP for a specific GTPase and cellular function are the same. In *C. elegans*, there are 30 genes encoding proteins harboring RhoGAP domains, and 18 of them are expressed in muscle. We obtained loss of function mutants for these 18 genes and conducted PAT-6 immunostaining. Mutations in 2 genes yield the same muscle boundary defect as *pix-1* mutants. Therefore, our results provide the first evidence that a PIX protein has an important function in muscle, and we have identified the first GAP protein for the PIX pathway.

P1568/B703

Mechanisms Regulating the Differential Adhesion and Spreading Characteristics of Hela Cells, Seeded on Fibronectin, Galectin 8 Or a Mixture of the Two.

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Galectin-8 is a mammalian lectin, which specifically binds to β-galactoside residues. It is secreted, and has the potential of modifying cell-extracellular matrixes interactions. Galectin-8 is widely expressed in various tissues, and has roles in vascular and lymphatic angiogenesis, B-cell function, and cancer metastasis. However, how galectin-8 affects cell spreading and adhesion is still poorly understood. Here, we describe the differential cytoskeletal and adhesion dynamics of HeLa cells, spreading on rigid flat substrate functionalized by fibronectin or galectin-8, or a mixture of both proteins. Using interference reflection microscopy, we found that HeLa cells, plated on galectin-8, have a faster spreading rate and a larger cell area than cells seeded on fibronectin. Measuring the detachment forces by Single Cell Force Spectroscopy revealed that the detachment forces per unit area on galectin-8 were two-fold higher than on fibronectin. On fibronectin, paxillin-positive focal complexes formed in a few minutes after seeding, matured into focal adhesions (FA) in about half an hour. On galectin-8, fuzzy clusters of paxillin at the periphery of lamellipodia underwent transformation into narrow strips associated with thin actin bundles perpendicular to the cell edge. They moved centripetally, and eventually vanished. During the spreading process, the total intensity of paxillin clusters increased on fibronectin, but decreased on galectin-8. On fibronectin, after half an hour of spreading, actomyosin forms circumferential fibres, while on galectin-8, at the same time, dynamic aster-like actomyosin structures assembled in the center of the cell. The total myosin-II filament intensity was weaker on galectin-8 than that on fibronectin. Furthermore, the addition of soluble RGD reduces cell spreading on fibronectin, but does not affect cell spreading on galectin, while the addition of thiodigalactoside does not change cell spreading on fibronectin, but reduces cell adhesion and spreading on galectin-8. The difference between cell spreading on fibronectin and galectin was associated with differential regulation of small Rho GTPase activity by cells seeded on these two ligands. We then studied how a mixture of fibronectin and galectin-8 modulates cell adhesion and spreading. Galectin coated substrates promoted formation of filopodia more effectively than fibronectin, and the maximal formation of filopodia was observed in cells seeded on substrates coated with a mixture of both proteins. We consider the possibility that galectin-8, at high concentrations interferes with integrin mediated signaling and blocks FAs and stress fiber formation, while at low concentrations galectin can enhance the adhesion and thereby cooperate with integrin mediated signaling.

P1569/B704

Spatiotemporal Regulation of Clathrin Containing Adhesion Complexes in Proliferating and Quiscent Cells.

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Clathrin-mediated endocytosis is the most extensively studied trafficking pathway from the cell surface to membrane-bound organelles. Cells exhibit two major modes of endocytic clathrin coats: dynamic

clathrin-coated pits and large, long-lived clathrin lattices called plaques. Recent studies show that clathrin plaques mediate cell adherence to the extracellular matrix via integrins and act as molecular scaffolds for transmission of mechanical forces required for migration. Canonical cell adhesion complexes (i.e. focal adhesions) are tightly regulated in proliferating and quiescent cells. However, it is not known how clathrin containing adhesion complexes (i.e. plaques) are regulated in cells. Using fluorescence live cell imaging and single particle tracking analysis, we observed significant variation in dynamics and spatial density of clathrin plaques across different stages of the cell cycle. Clathrin plaque density is high during G1 and G2 phases and low during S phase, exhibiting negative correlation with focal adhesion density. Furthermore, treating cells with Cyclin D1 inhibitor, which reduces focal adhesion maturation, increased plaque formation. We also found that clathrin plaque density gradually increased as cells exit the cell cycle, peaked in quiescent cells, and reduced back upon stimulating cell cycle re-entry. These results show that clathrin plaques provide cell adhesion in coordination with focal adhesions and their cooperation is tightly regulated in proliferating and quiescent cells.

P1570/B705

Identification of Context-specific Force-sensitive Protein Complexes within Focal Adhesions.

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Mechanical cues, such as applied loads or extracellular matrix (ECM) stiffness, activate specific cell signaling pathways to affect key cellular processes, such as migration and differentiation. Cellular detection of these physical cues is thought to be mediated by protein conformation changes and the formation of key force-induced protein complexes within focal adhesions (FAs), subcellular structures that mediate physical connections between the ECM and force-generating cytoskeleton. As the key constituents that comprise force-induced protein complexes are unknown, we developed a novel technique to identify them by probing for proteins, or phosphorylation states, that selectively localize to areas of high tension. We achieve this by combining a FRET-based molecular tension sensor that reports the loads across a key mechanical linker protein, vinculin, with standard immunofluorescence protocols to probe for protein localization and phosphorylation states. Using this technique, we identified ten FA proteins and six phosphorylation states that are preferentially and specifically recruited to areas of high vinculin tension, suggesting that they form different force-induced protein complexes. Furthermore, as FAs are diverse structures that contain vinculin in various biochemically-distinct and mechanically loaded states, we hypothesized that differential force-induced protein complexes form in various FA subclasses. To test this idea, we developed a machine learning algorithm to identify two FA subclasses, those associated with stress fibers or protrusions, and searched for distinct vinculin-based force-induced protein complexes. Interestingly, nine proteins and two phosphorylation states exhibited differential force sensitive protein complex formation based on their associated FA class. In total, this work develops and uses a new imaging-based approach to demonstrate the existence of context-specific force-induced signaling capabilities amongst different FA proteins. Our observation that vinculin forms a variety of force-induced protein complexes in various FA subclasses suggests that vinculin load may activate different mechanosensitive signaling pathways in various adhesions. Indeed, preliminary experiments suggest that force-induced protein complexes that form in different FA subclasses are required for directed migration in response to aligned ECM.

P1571/B706

Spatio-temporal Dynamics between BMP Receptor and Integrin to Control Cell Decision Making.

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Understanding how cells integrate multiple signals from the extracellular matrix to make a decision, achieve specific cell differentiation and acquire cell identity is a challenging question in cell biology. We have previously shown that integrins and BMPR are able to cooperate for the cell response to biophysical cues from the microenvironment. Whether the temporal dynamics of both receptors are coupled to their spatial gradients to guide pivotal intracellular processes and to generate diversity in cell response was the pending question. Our goal is to explore whether cell fate depends on the differential spatio-temporal distribution between BMP receptor and Integrins. Based on biomaterials, live cell imaging and optogenetic tools, we show that the specificity of cellular response to receptor stimulation is encoded by their spatial and temporal dynamics. First the cooperation between BMPR and integrins depends on a discrete organization into segregated spatial domain between BMPR and integrins at the cell surface. Second the temporal control between BMPR and integrins is sufficient to drive cell spreading, cell mechanics and cell differentiation. Finally we demonstrate that integrins and BMP receptors finely communicate in space and time to control cell decision making.

P1572/B707

Vitronectin Promotes Axon Specification of Cerebellar Granule Cells Via αvβ5 Integrin and Pi3-kinase/gsk3β Pathway.

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Mouse cerebellar granule cells (CGCs) are the most numerous cells in cerebellum. There are still many unknown parts in cerebellar development, the generation of CGCs is also one of the mysteries of cerebellar development. Vitronectin (VN), one of the extracellular matrix proteins, has been reported to be expressed in the developing cerebellum. We previously revealed that VN controls the maturation of CGCs through the promotion of the initial differentiation stage progress. αvβ5 integrin is a receptor for VN in CGCs and is involved in the progression of the initial differentiation stage in CGCs. In this stage, axon specification and progress of the initial differentiation has been known to happen simultaneously. This finding motivated us to address the function of VN on the axon specification of CGCs. In addition, previous studies have shown that the PI3K / GSKβ pathway is involved in axonal formation in the hippocampus. Therefore, we hypothesized that VN and αvβ5 integrin are involved in the axon specification of CGCs. The aim of our research is to confirm that VN and its receptors are involved in the axon specification of CGCs, and to show the involvement of the PI3K / GSK3β pathway. First, we analyzed the effects of VN on the axon specification of CGCs in VN knockout mice-derived CGCs. Immunofluorescent staining showed that the loss of VN increased the ratio of CGCs with no axon, and VN addition increased the ratio of CGCs with multiple axons. Next, to analyze whether αvβ5 integrin is served as a VN receptor in the axon specification of CGCs, the effect of β5 integrin knockdown on VN-dependent axon specification was examined. The knockdown of β5 integrin failed to up-regulate the ratio of cells with multiple axons by VN addition. Furthermore, to examine the signal pathway of the

axon specification, we analyzed the effect of PI3K and GSK3β inhibitors on the VN-dependent axon specification. Wortmannin, a PI3K inhibitor, suppressed multiple-axon formation. Similarly, LiCl, a GSK3β inhibitor, promoted multiple axon-formation. In addition, we observed that the addition of VN to Neuro2a cells up-regulated the phosphorylation level of GSK3β using Western blotting. Taken together, our results indicate that VN promotes the axon specification of CGCs via αvβ5 integrin and PI3K / GSK3β pathway in the developing cerebellum.

P1573/B708

An In-depth an alysis of Cardiomyocyte Rigidity Sensing through Quantitative Imaging of Cardiomyocyte Forces and Integrin Mechanosensing Using PDMS Nanopillar and DNA Origami Nanoarrays.

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Mechanical properties are cues for many biological processes in health or disease. In the heart, changes to the extracellular matrix composition and cross-linking results in stiffening of the cellular microenvironment during development. Moreover, remodelling after myocardial infarction, or in cardiomyopathies lead to fibrosis and a stiffer environment. Previous studies established a direct relationship between rigidity and the contractile forces of cardiomyocytes. However, it is still elusive how cardiomyocytes sense matrix stiffness. By combining nanopillar arrays, PDMS gels with defined stiffness and FRET molecular tension sensors, we identify here a fundamental mechanism for cardiomyocyte rigidity sensing, whereby single cardiomyocyte adhesions sense simultaneous (fast oscillating) cardiac and (slow) non-muscle myosin contractions. Together this leads to oscillating tension on the mechanosensitive adaptor protein talin on substrates with a stiffness of healthy adult heart tissue, compared to no tension on embryonic heart stiffness and continuous stretching on fibrotic stiffness. To further investigate the mechanosignalling pathways downstream of cardiomyocyte integrins, we designed DNA origami nanoarrays, which enable the display of receptor ligands in a highly customisable manner, with modifiable parameters including ligand number, ligand spacing, inter-cluster spacing and multivalency. Combining the nanoarrays with various imaging techniques (FRET/FLIM, FRAP, SIM), we find distinct clustering behaviour of cardiomyocyte integrins as well as the involvement of additional cytoskeletal proteins in the regulation of cardiomyocyte rigidity sensing.

P1574/B709

Tenascin-C Evokes Phenotypic Changes in Human Mammary Fibroblasts to Myofibroblasts with High Contractility Via Integrin αvβ1/TGF-β/SMAD Signaling Axis.

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Tenascin-C (TNC) is strongly expressed in the fibroblasts and cancer cells of breast cancer tissues. We examined phenotypic changes in human mammary fibroblasts (HMFs) after a treatment with TNC. The addition of TNC induced the aggregated cluster formation of cultured HMFs, similar to the “hill-and-valley” appearance of smooth muscle cells. The protein expression of α-smooth muscle actin (SMA) and calponin was significantly up-regulated. TNC increased the number of α-SMA- and/or calponin-positive cells with well-developed stress fibers in immunofluorescence, which enhanced contractile ability in the collagen gel contraction assay. TNC significantly up-regulated itself at the mRNA and protein levels, whereas the gene expression of *COL1A2*, *COL3A1*, *and FN1* remained unchanged. Double

immunofluorescence of human breast cancer tissues showed α-SMA and/or calponin-positive myofibroblasts in the TNC-deposited cancer stroma. Among several receptors for TNC, the protein levels of the αv and β1 integrin subunits significantly increased after the TNC treatment, while the gene expression of β3 significantly decreased. Immunofluorescence showed the augmented co-localization of αv and β1 at focal adhesions after the TNC treatment. Immunoprecipitation using an anti-αv subunit antibody revealed a significant increase in co-precipitated β1 levels with TNC in lysates. The addition of TNC induced the phosphorylation of SMAD2/3, and the nuclear translocation of SMADs was observed. SB-505124 and SIS3 blocked myofibroblast differentiation by TNC. Thus, TNC enhances its synthesis by forming a positive feedback loop and increases the levels of integrin αvβ1 heterodimers, which are known to activate TGF-β signaling. This was followed by a change to highly contractile myofibroblasts. TNC may contribute to the cancer stroma formation characteristic of breast cancer tissues.

P1575/B710

Galectin-8 Mediates Cyclosporine-induced Fibrogenesis in Human Gingival Fibroblasts.

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Galectins are glycan-binding proteins that modulate a variety of cellular processes including fibrosis in several organs. Galectin-8 (Gal-8), one of the most widely expressed galectins, has two carbohydrate recognition domains (CRDs) through which can bind and activate selected β1-integrins and transforming growth factor-beta receptor TGFβRI, crucially involved in fibrogenesis. We recently described that Gal-8 induces partial epithelial mesenchymal transition (EMT), which included an increased expression of fibronectin, as is characteristic of fibrogenesis. Here we study the role of Gal-8 in the response of gingival connective tissue cells to cyclosporine, which unwanted effects include gingival overgrowth and disruption of gingival connective tissue homeostatic balance, leading to pathogenic fibrosis. In human gingival fibroblasts (HGF), we show that Gal-8 stimulates the expression of type I collagen and fibronectin, potentiates the expression of CTGF induced by TGF-β, interacts with α5β1 integrin and type II TGF-β receptor, activates β1-integrins and stimulates Focal adhesion kinase (FAK) but not Smad2/3 signaling. Cyclosporine and the proinflammatory tumor necrosis factor alpha both increased Gal-8 expression. Experiments in NIH3T3 fibroblasts silenced for Gal-8 with siRNA, demonstrated that cyclosporine-induction of fibronectin expression requires Gal-8. Taken together, these results reveal Gal-8 as a fibrogenic stimulus involving β1-integrins/FAK pathways in human gingival fibroblasts. Gal-8-mediated fibrogenesis can be triggered by cyclosporine and, afterwards, might be exacerbated by the inflammatory condition that usually accompanies a fibrotic process. Thus, Gal-8 emerged as mediator of cyclosporine-induced fibrogenesis and can offer new opportunities to ameliorate an unfortunate collateral problem of this otherwise highly effective immune-suppressive drug. (Grants FONDECYT # 1170555, FONDECYT, FONDECYT # 1131122, Comisión Nacional de Investigación Científica y tecnológica (CONICYT) Basal Program No. AFB-170005).

P1576/B711

Alpha 6 Integrins Regulate the Expression of Laminin-511 and Cxcr4 to Promote Endothelial Tubular Morphogenesis.

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During angiogenesis, endothelial cells engage components of the extracellular matrix through integrin-mediated adhesion. Endothelial cells express laminin-411 and laminin-511 that bind to integrins, including the α6 integrins, α6β1 and α6β4. However, little is known about the contribution of these laminins to endothelial tubular morphogenesis and stability. We used two organotypic angiogenesis assays in conjunction with RNAi approaches to demonstrate that endothelial depletion of either the α4 chain of laminin-411 or the α5 chain of laminin-511 inhibited sprouting and tube formation. Depletion of α6 integrins resulted in similar phenotypes. Interestingly, depletion of α6 integrins also inhibited the expression of laminin-511, which correlated with the loss of tubular stability. Loss of either α6 integrins or laminin-511 resulted in the inhibition of the expression of CXCR4, a gene previously associated with sprouting endothelial cells. Pharmacological inhibition of CXCR4 signaling suppressed endothelial sprouting and morphogenesis, suggesting that the interaction of α6 integrins and laminin-511 regulate of endothelial tubular morphogenesis in part by regulating the expression of CXCR4. Taken together, our results suggest that α6 integrins regulate gene expression to promote both early events in tubular morphogenesis, as well as the stability of established endothelial tubes.

P1577/B712

Ultra Dilution of *Rhus Toxicodendron* Induced Cell Adhesion in Mouse Preosteoblast Cell Line.

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Background: Ultra dilution of *Rhus toxicodendron* (*R. Tox*) has been used as a homeopathic remedy and treatment of anti-inflammatory conditions. Previously, we reported that *R. Tox* showed modulate the inflammation in mouse chondrocyte and preosteoblastic MC3T3-e1 cell line. During the inflammation process, cells adhere to the extracellular matrix (ECM) and then migrate to the inflammation site. Here we examine the cell adhesion process after treatment of *R. Tox* using MC3T3-e1 cells. **Methods:** the cells are stimulated with *R. Tox*, cell adhesion improved comparing with fibronectin (FN) and gelatin stimulations. To access the cell adhesion assay, cells are starved, cultured and detached using EDTA containing media. Adhesive cells were measured using WST(water-soluble tetrazolium salt)-8 assay. To examine the intracellular signals after stimulation of *R. Tox*, the tyrosine phosphorylation of FAK, Src, and Paxillin analyzed and identified phosphorylation of FAK, Src, and Paxillin using phosphor-specific antibodies by immunoblot assay. The focal adhesion formation examined by immunocytochemistry assay **Results:** the cell adhesion increased after stimulated with *R. Tox* (FN:20.50%, *R. Tox*:44.80%, Gelatin:17.11%). The tyrosine phosphorylation of FAK was increased compare with FN and gelatin, and also Paxillin and Src phosphorylation when stimulating with *R. Tox*. Additionally, *R. Tox*-stimulated MC3T3-e1 cells also formed focal adhesions. **Conclusions:** *R. Tox* has roles of ECM like FN which has an Arg-Gly-Asp (RGD) motif and then transduces the extracellular signals into intracellular.

P1578/B713

Investigating Integrin α3β1-mediated Regulation of Transcription Factor Brain-2 (brn-2/ Oct-7/ N-oct3) in Breast Cancer Cell Invasion.

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Tumor cells receive extracellular signals from the tumor microenvironment that play a critical role in regulating gene expression that drives malignant progression and invasion; however, roles for integrins in this process remain underexplored. The laminin-binding integrin, α3β1, is a heterodimeric cell surface protein that promotes tumor growth, progression and metastasis in murine models of breast cancer, and our lab and others have demonstrated a role for α3β1 in promoting breast cancer cell invasion. To explore mechanisms of α3β1-mediated effects on cell invasion, we mined data from our previously published microarray analysis in which we compared the transcriptomes of MDA-MB-231 cells (a triple-negative human breast cancer cell line) that express either non-targeting shRNA (control cells) or α3-targeting shRNA (α3-knockdown cells). We showed that knockdown of α3 leads to the suppression of the mRNA encoding the transcription factor, Brain-2 (BRN-2). BRN-2 is a master regulator of invasive phenotype in melanoma; however its role in breast cancer is unknown. We hypothesize that integrin α3β1-dependent regulation of BRN-2 expression in breast cancer cells promotes invasion. Here we confirm that RNAi-mediated targeting of α3β1 in MDA-MB-231 cells reduces BRN-2 mRNA and protein levels. Additionally, microarray data confirm downregulation of several predicted BRN-2 target genes in α3-knockdown cells compared to control cells. To investigate if integrin α3β1 regulates BRN-2 gene transcription we are performing BRN-2 promoter reporter assays in control and α3-knockdown cells. RNAi-mediated suppression of BRN-2 in MDA-MB-231 cells followed by Transwell invasion assay showed decreased invasion of BRN-2 knockdown cells, confirming a pro-invasive role for BRN-2 in these cells. In summary, our work identifies a novel role for integrin α3β1 in the regulation of BRN-2, revealing a potential mechanism of gene regulation that affects breast cancer cell invasion.

P1579/B714

Investigating the Roles of Laminin-binding Integrins in Breast Cancer Cell Invasion.

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Integrins are αβ heterodimeric receptors on the cell surface that mediate cell adhesion to the extracellular matrix and are involved in cell signaling. The laminin-binding integrin α3β1 is expressed in all epithelial tissues, including the epidermis of skin and the breast epithelium, where it has important roles in regulating cell survival, migration and proliferation. Our group and others have previously linked α3β1 signaling and adhesion functions to tumor growth, invasion and metastasis in models of skin and breast cancer. Two other laminin-binding integrins, α6β1 and α6β4, have also been implicated in the regulation of cancer cell invasion. To investigate roles of α3β1 in cell invasion, and to explore its potential interactions with α6 integrins, we used a genetic approach of CRISPR technology to knock out the ITGA3 gene (encoding the integrin α3 subunit) in the triple-negative breast cancer cell line, MDA-MB-231 (i.e., α3KO cells). Flow cytometry confirmed complete loss of α3β1 expression from the cell surface of α3KO cells. However, we observed a shift in surface expression of α6 integrins, where α6β4 levels decreased while levels of α6β1 appeared to increase. This shift presumably resulted from partnering of the α6 subunit with excess β1 subunit following the CRISPR-mediated ablation of α3. As α6β1 has also been shown to promote cell invasion in breast cancer cells, we next tested whether or not the increased surface levels of α6β1 in α3KO cells partially compensates for the loss of α3β1 during cell

invasion. Towards this goal, we used siRNA to target the ITGA6 mRNA (encoding the integrin α6 subunit) in α3KO cells, or in parental MDA-MB-231 cells as a control, then compared invasion using Transwell Matrigel assays. Initial results showed that loss of either the α3 or α6 integrin subunits alone reduced migration to a similar extent, and we did not observe a synergetic effect upon loss of both α3 and α6 subunits. These results suggest that α3β1 and α6 integrins both promote cell invasion, but that neither alone is sufficient in MDA-MB-231 cells to achieve baseline invasion levels. Studies are underway to determine whether α6 integrins can compensate for the loss of α3β1 with regard to other α3β1-dependent functions that we have identified in MDA-MB-231 and other breast cancer cells.

P1580/B715

Cell-ECM Attachment Facilitates Inflammatory Signaling by Suppressing Interleukin-1 Receptor Endocytosis.

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Interactions between cells and their extracellular matrix (ECM) mediate a plethora of cellular processes such as development and tissue repair. Such interactions are largely mediated by focal adhesions - integrin-based adhesion complexes that enable cells to sense immobile cues and correctly respond to mechanical properties of their microenvironment. In addition, focal adhesions are also known to mediate responses to soluble cues such as interleukin-1 (IL-1), yet the mechanism is unknown. Here we show that focal adhesions indirectly regulate IL-1 receptor (IL-1R) surface expression and IL-1 signaling through modulation of cortical tension. To test whether focal adhesions are important for IL-1 signaling, we plated primary human fibroblasts on surfaces that either suppressed or promoted focal adhesion assembly. We found an increase in both IL-1 receptor surface expression and downstream signaling events in cells that assembled focal adhesions, suggesting that focal adhesions are indeed important for IL-1 signaling. Interestingly, we found that IL-1 signaling can also be suppressed by pharmacological perturbations that attenuate myosin contractility without affecting integrin signaling and focal adhesion assembly, suggesting an indirect regulation of IL-1 signaling by focal adhesions. Thus, we hypothesized that cortical tension, a factor that focal adhesions contribute to, may play a role in regulating IL-1 signaling. To test whether cortical tension modulates IL-1 signaling through IL-1R surface expression, we locally modulated cortical tension by plating cells on patterned surfaces of defined shapes and showed that IL-1R is consistently enriched in areas of high cortical tension. Furthermore, inhibition of endocytosis abolished heterogenous receptor distribution and rescued IL-1 downstream signaling events in cells with suppressed cortical tension, suggesting that cortical tension modulates IL-1 signaling through endocytosis of its surface receptor. To understand the biological role of cortical tension-regulated IL-1 signaling, we plated cells on ECMs of different stiffness that are physiologically and pathologically relevant. By combining immunofluorescence and biochemical assays, we found that both receptor expression and IL-1 signaling are significantly upregulated on the stiffer ECM. Since cells in pathological environments, such as fibrotic tissues, are often under high cortical tension, our results provide insight into a possible molecular link between mechanical properties of cell microenvironment and inflammatory signaling.

P1581/B716

The LTB_4-BLT1 Signaling Axis Coordinates Actomyosin Dynamics and Beta-2 Integrin Trafficking to Drive Intravascular Neutrophil Response to Infection.

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The eicosanoid Leukotriene B_4 (LTB_4), relays chemotactic signals to direct neutrophil interstitial migration in response to injury through its receptor, BLT1. However, whether the LTB_4-BLT1 axis relays signals during intravascular neutrophil response has not been directly addressed. To test this issue, we developed a sterile bacterial infection model in the mouse footpad and used intravital microscopy to directly visualize the impact of LTB_4-BLT1 axis on the intravascular neutrophil dynamics. Here, we report that LTB_4 produced by neutrophils acts as an autocrine/paracrine signal via BLT1 to drive their recruitment, arrest, and extravasation during infection in living mice. To understand the mechanism behind these findings, we utilized Intravital Subcellular Microscopy (ISMic) and discovered that LTB_4 elicits cell adhesion and polarization during neutrophil arrest in *vivo*. Specifically, LTB_4 signaling coordinates the dynamic redistribution of - 1) non-muscle Myosin IIA (NMIIA) to the back of cells, and 2) $ß_2$-integrin (Itgb2) to the neutrophil-endothelial interface, whose retention on the cell surface promotes neutrophil arrest and subsequent extravasation. Additionally, using an in *vitro* adhesion assay, we found that blocking LTB_4-BLT1 signaling or NMIIA activation results in a reduction of Itgb2 recycling to the plasma membrane in primary human neutrophils. Notably, we also discovered that neutrophils shed extracellular vesicles (EVs) in *vivo* and that blocking their biogenesis/release prevents the autocrine/paracrine actions of LTB_4 signaling therefore inhibiting neutrophil arrest and extravasation response. Overall, our study unravels a crucial function for LTB_4 in promoting neutrophil communication in the vasculature during early response to infection.

P1582/B717

Using Phosphoproteomics to Decipher Syndecan-3 Mediated Signaling Events in Myoblasts.

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Muscle satellite cells are quiescent muscle progenitors residing within the muscle tissue which are required for skeletal muscle maintenance and regeneration. The signalling networks that regulate satellite cell fate decisions are not fully understood. Particularly it is still unclear how satellite cells are maintained quiescent, but it is thought that proteoglycans found within the satellite cell niche play a critical role. Previous studies have shown that genetic ablation of the transmembrane proteoglycan syndecan-3 in mice dramatically improves muscle health and regenerative potential in ageing and in pathological conditions such as muscular dystrophy. Primary myoblasts from syndecan-3 null mice show a global upregulation of phosphorylated-tyrosine. Therefore, we hypothesised that syndecan-3 is involved in the regulation of several signalling pathways simultaneously. To test this hypothesis, we used a phosphoproteomics-based approach to identify proteins that are differentially phosphorylated when syndecan-3 expression is knocked-down in proliferating myoblasts. We then employed functional enrichment analysis to identify the signalling pathways that were affected by syndecan-3 knockdown in myoblasts. Pathway enrichment analysis identified insulin/PI3K/mTOR as one of the pathways regulated by knock-down of syndecan-3. Results from insulin stimulation experiments in C2C12 and primary satellite cell-derived myoblasts further confirmed that syndecan-3 is a regulator of the insulin signalling

pathway, likely in an AKT-dependant mechanism. These results indicate that syndecan-3 regulates the balance between myoblast proliferation and differentiation.

Cell Death: Alternative Death Pathways

P1583/B719

Pannexin-1 Limits the Production of Proinflammatory Cytokines during Necroptosis.

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The activation of mixed lineage kinase-like (MLKL) by receptor-interacting protein kinase-3 (RIPK3) controls the execution of necroptosis, a regulated form of necrosis that occurs in apoptosis-deficient conditions. Active oligomerized MLKL triggers the exposure of phosphatidylserine residues on the cell surface and disrupts the plasma membrane integrity by forming lytic pores. MLKL also governs endosomal trafficking and biogenesis of small extracellular vesicles as well as the production of proinflammatory cytokines during the early steps of necroptosis, however the molecular basis continues to be elucidated. Here, we find that MLKL oligomers activate Pannexin-1 (PANX1) channels, concomitantly to the loss of phosphatidylserine asymmetry. This plasma membrane “leakiness” requires the small GTPase RAB27A and RAB27B isoforms, which regulate intracellular vesicle trafficking, docking and fusion with the plasma membrane. Although cells in which PANX1 is silenced or inhibited normally undergo necroptotic death, they display enhanced production of cytokines such as interleukin-8, indicating that PANX1 may tamper with inflammation. These data identify a novel signaling nexus between MLKL, RAB27, and PANX1, and propose ways to interfere with inflammation associated with necroptosis.

P1584/B720

Reversal of Ferroptotic Cell Death Process by an astasis.

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Anastasis (Greek for “rising to life”) is a natural cell survival mechanism to allow recovery of dying cells from the brink of death. The classical view of programmed cell death has long assumed that once initiated the dying process is irreversible. Overturning this general assumption, our earlier studies demonstrated that the apoptotic form of programmed cell death is reversible. This observation on apoptosis reversal raises an intriguing question: which forms of programmed cell death can be reversable? by time-lapse live-cell microscopy, we made a new discovery that ferroptosis, the non-apoptotic iron-dependent form of programmed cell death, is also reversible. Ferroptotic cell death can be induced by natural stimuli such as the neurotransmitter glutamate, or by synthetic agents such as the small molecule erastin. Unlike apoptosis reversal, removal of ferroptosis inducers is insufficient to allow ferroptotic dying cells to reverse the initiated cell death process such as lipid peroxidation. However, by removing the cell death inducer and also restoring the intracellular reducing environment with pharmacological approach such as by providing the reduced form of glutathione or the radical-trapping antioxidant ferrostatin-1, ferroptotic dying cells can be rescued and promoted to recover. Interestingly, although potent ferroptotic inhibitors such as aminooxyacetic acid, deferoxamine, dopamine, and vitamin C can prevent initiation of ferroptosis, added alone they are unable to rescue the ferroptotic dying cells. This indicates the regulatory distinctions between preventing and reversing ferroptosis.

Taken together, these results demonstrate that ferroptosis is reversible, and suggest strategies to mediate the reversibility of ferroptosis, with physiological, pathological, and therapeutic potentials in which involvements of ferroptosis are beginning to emerge.

P1585/B721

Gallic Acid Triggers Iron-dependent Apoptotic, Ferroptotic and Necroptotic Cell Death Mechanism.

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Gallic acid (GA) is a natural anticancer phenolic compound that can be found in many food sources including edible mushrooms, fruits, herbs, teas and vegetables. Studies generally attribute the anticancer activity of GA to the induction of apoptosis. Interestingly, we found that GA activates apoptotic, ferroptotic and necroptotic mechanisms simultaneously in the same cancer cells. By time-lapse live-cells confocal microscopy, we discovered that GA can trigger multiple types of cell death pathways including apoptosis characterized by mitochondrial cytochrome *c* release to cytosol and caspase-3 activation, ferroptosis by lipid peroxidation, and necroptosis by MLKL activation and loss of plasma membrane integrity. By pharmacological approach, we found that this form of cell death can be suppressed by iron chelator such as deferoxamine (DFO), indicating that it is an iron-dependent cell death. However, this GA-induced cell death cannot be suppressed by apoptosis inhibitor such as the potent pan-caspase inhibitor Z-VAD-FMK, ferroptosis inhibitors such as ferrostatin-1 (Fer-1) and aminooxyacetic acid (AOA), as well as necroptosis inhibitors such as necrostatin-1 (Nec-1) and MLKL inhibitor necrosulfonamide (NSA), when they are applied either alone or in combinations. This indicates that GA can also trigger the unidentified cell death mechanism(s), which is mediated by an iron-dependent pathway. Interestingly, NSA exerts a synergistic effect by enhancing the sensitivity of various cancer cells upon GA induction. Taken together, these results suggest potential new strategies to enhance the efficacy of this natural anticancer substance, and also provide a useful model for studying the crosstalk between apoptosis, ferroptosis, and necroptosis.

P1586/B722

Unc-53/nav2 Inhibits the Phagocytosis of Apoptotic Cells in *Caenorhabditis Elegans*.

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The nematode *Caenorhabditis elegans* has been used to unravel the signalling processes controlling apoptosis and cell corpse engulfment. The engulfment of apoptotic cells is mediated by three signalling pathways: (i) CED-1/SREC, CED-6/GULP, CED-7/ABC1, DYN-1/Dynamin and (ii) CED-2/CrkII, CED-5/DOCK180, CED-10/RAC, CED-12/ELMO and (iii) ABI-1/ABI and ABL-1/ABL. CED-1,6,7 activate DYN-1 to control membrane dynamics while CED-2,5,12 activate CED-10 to altered actin polymerization. ABI is an adaptor protein for the oncoprotein ABL and participates in cell adhesion and migration as well as cancer signalling. ABI-1 is required for ABL-1 mediated suppression of cell corpse engulfment. ABI-1 interacts physically and genetically with the protein UNC-53 to control cell migration and actin-mediated outgrowth. UNC-53 is a cytoskeletal binding protein homologous to the mammalian Neuron Navigator genes (NAV-1,2,3), a gene family with diverse functions including contributions to the growth and development of tumorigenic cells. In this study we used *C. elegans* to examine the role of UNC-53 in apoptotic cell corpse engulfment and the related process of distal tip cell (DTC) migration. We constructed double mutants between loss of function *unc-53* mutants and mutant *ced-1*, *ced-2*, *ced-5*,

and *ced-10* animals. *unc-53* suppressed cell corpse engulfment and distal tip cell migration of *ced-1*, *ced-2*, and *ced-10* but not *ced-5* mutants, placing *unc-53* outside of a *ced-1* pathway but not entirely independent of *ced-2* and *ced-5*. To determine if *unc-53* is involved in apoptosis directly or through engulfment we examined *unc-53* alongside *ced-3*/Caspase mutants and found that *unc-53* does enhance *ced-3* and is likely not involved in apoptosis directly. *unc-53* also does not affect the timing, number or morphology of cell corpses, arguing for a role in engulfment processes. We also show that *unc-53* is expressed after the comma stage, when programmed cell death is complete, suggesting a role for *unc-53* in engulfing rather than engulfed cells. In the process of DTC migration *unc-53* strongly suppresses the cell guidance defects of *ced-10* and *mig-2*/Rho by eliminating anteroposterior polarity reversals. Our current model is that *unc-53* suppresses cell engulfment in a pathway through its physical interactions with ABI-1, possibly through its activity in cell polarity decisions.

P1587/B723

Necrosome-mediated Control of Immune Response by Canonical Nf-kb Activation.

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Unlike apoptotic cell death, the release of inflammatory mediators called damage-associated molecular patterns (DAMPs) from necroptosis promotes inflammation and innate immune responses. However, its ability to regulate adaptive immune responses as well as the underlying mechanism, remains largely unknown. Here, in RIP3 expressing cells and FADD null cells, we found that TNF induced persistent activation of NF-κB signaling cascades under the condition of necroptosis, but not of apoptosis independently of DAMPs liberation. Furthermore, we observed that TNF-induced necroptosis resulted in drastic up-regulation of pro-inflammatory cytokines, and such enhanced cytokine expression was completely abrogated by pharmacological inhibition or genetically abolishment of IKKβ. An alysis of the signaling molecules composed of necrosome revealed that upstream NF-κB components were associated with receptor interacting protein kinase 1 (RIP1)-RIP3 complex under the necroptotic conditions. Together, these results suggest that necroptosis up-regulates autonomous cytokine production by canonical NF-κB activation, and thus our study uncovers the signaling crosstalk between necroptosis and inflammatory responses to elicit adaptive immune response.

P1588/B724

Rip3-independent Function of Mlkl in Trail-induced Cancer Cell Death.

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Mixed Lineage Kinase Domain-like (MLKL) is the essential molecule of programmed necrotic cell death, Necroptosis. To induce necroptosis, MLKL is phosphorylated by upstream partner, Receptor Interacting Protein Kinase-3 (RIPK3 or RIP3) and then translocate cellular membrane to disrupt membrane integrity. Recent data suggests that function of MLKL in RIP3-indendent manner in the effective generation of intraluminal and extracellular vesicles as well as in myelin sheath breakdown to promotes sciatic nerve regeneration. In this study, we investigated that whether MLKL play a role in link between TRAIL receptor endocytosis and apoptosis. Depletion of MLKL enhances TRAIL-induced cell death in various cancer cell lines and enhancement of cell death is RIP3-independent manner. TRAIL-induced cytotoxic signals are further increased by preventing endocytosis and MLKL depletion facilitates degradation of TRAIL-DR5. TRAIL-induced intracellular downstream signals were prolonged by depletion of MLKL and

then prolonged cytotoxic signals promote cell death. Our data indicate that defect of the intracellular trafficking of TRAIL-DR5 by depletion of MLKL enhances cancer cell death. Based on our findings, it is tempting to speculate that to promote death receptor signals, it may effective to reduced MLKL expression and to prevent survival receptor signal, upregulated MLKL expression contribute to abolish the intracellular signal through the MLKL-dependent extracellular vesicles secretion. Although the processes that regulate these pathways require further elucidation for detail molecular mechanisms, both ways will be the potent therapeutic strategies to target cancer.

P1589/B725

Waiting to Die: Nucleation-limited Signalosome Assembly Renders Human Cell Fate Decisions Inevitable.

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Multiple signaling proteins of the innate immune system exert their cellular activities by assembling into large macromolecular complexes known as signalosomes. We previously discovered that two such proteins - the pyroptosome scaffold ASC, and the antiviral signaling protein MAVS - each assemble through self-templating polymerization that is reminiscent of infectious protein particles known as prions (Cai et al. 2014; PMID: 24630723). More recently we revealed that prion-like activity broadly arises from structurally encoded kinetic barriers to nucleation - the probabilistic formation of a self-templating multimer de novo (Khan et al. 2018; PMID: 29979963). For proteins like MAVS and ASC, the nucleation barrier is so high that their soluble inactive states persist despite physiological concentrations that are highly supersaturated with respect to the assembled active state. To identify other innate immune signaling proteins that may function in this manner, we used DAmFRET, a flow cytometric cell-based assay of nucleation barriers, to screen 138 candidate prion-like modules from 129 human proteins that broadly function in programmed cell death and innate immune signaling. We discovered 36 of these proteins that are inherently capable of supersaturation and switch-like self-templating activation in living cells. We have further discovered a network of nucleating interactions between them, wherein polymerization of one protein nucleates the polymerization of specific additional proteins, resulting in a cascade of irreversibly activating protein switches. This widespread kinetic control over cell fate indicates that cells are literally waiting to die -- pyroptosis, necroptosis, and alternative cell fates downstream of these proteins are thermodynamically favored, and therefore inevitable with time. Surveying known disease-associated mutations in one such signalosome, we show that the nucleation barrier for BCL10 is balanced between hypo-activation and immunodeficiency on the one hand, and hyper-activation and lymphoma on the other. Finally, I will discuss our investigations into the implications of this phenomenon for aging-associated inflammation and innate immune memory in human monocytes.

P1590/B726

A Novel Ca^{2+}Influx Pathway during Clearance of Apoptotic Cells.

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A tons of apoptotic cells are generated by diverse stimulations in adults everyday. However, phagocytes clear apoptotic cells quickly and efficiently. This process is named efferocytosis. Successful efferocytosis can maintain tolerance of immune responses through secretion of anti-inflammatory cytokines such as

IL-10 and TGF-beta[1)]. If apoptotic cells are not removed accurately, they undergo secondary necrosis and damage to neighboring normal cells. Finally, it evokes serious diseases such as autoimmune disease and atherosclerosis etc[2,3)]. As a result, exquisite efferocytosis is important to prevent from abnormal immune responses. Although many factors related with efferocytosis have been studied, all mechanisms are not fully understood. Ca^{2+} is associated with many cellular functions including proliferation, migration, cytoskeleton rearrangement and phagocytosis[4)]. In addtion, it has revealed that extra/intracellular Ca^{2+} is important to engulf apoptotic cells[5,6)]. However, all mechanisms associated with Ca^{2+} influx during efferocytosis is elusive. We found that interaction between Stim and Orai is enhanced during clearance of apoptotic thymocytes. This interaction is specific to apoptotic cells, not live thymocytes. In addition, apoptotic cell itself induced the interaction between Stim and Orai. Overexression of Stim and Orai increased engulfment of apoptotic cells. These results suggest a novel Ca^{2+} influx pathway during efferocytosis. Our findings will help to understand efferocytosis in detailed and develop a new drug for diseases generated by defect of efferocytosis. **References** 1)Fadok VA *et al.* Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J Clin Invest*. 101: 890-898. (1998) 2)Elliott MR and Ravichandran KS. The Dynamics of Apoptotic Cell Clearance. *Dev Cell.* 25;38(2):147-60. (2016) 3)Yurdagul a Jr *et al.* Mechanisms and Consequences of Defective efferocytosis in Atherosclerosis. 8:4:86. (2017) 4)Putney, J.W. A model for receptor-regulated calcium entry. *Cell Calcium* 7. 1-12 (1986). 5)Gronski M. A. *et al*. An essential role for calcium flux in phagocytosis apoptotic cell engulfment and the anti-inflammatory response. *Cell Death Differ*. 16(10): 1323-1331 (2009). 6)Wang Y *et al.* Mitochondrial Fission Promotes the Continued Clearance of Apoptotic Cells by Macrophages. *Cell.* 5;171(2):331-345.e22. (2017)

P1591/B727

Mitochondrial Targeting Sequence of Telomerase Reverse Transcriptase Acts as a Cell Death Regulator.

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Telomerase reverse transcriptase TERT is a major component of telomerase that elongates telomere region at chromosomal ends. Recently, TERT has been reported to localize also in mitochondria that lack telomere region. Based on the observation that TERT accumulates in mitochondria under oxidative stress, mitochondrial TERT has been predicted to participate in the regulatory process of apoptotic cell death. However, the role of mitochondrial TERT in the process remains controversial mainly because of the absence of information about the real-time dynamics of TERT localization. Here, we developed a new TERT probing method that did not disturb TERT activity and localization. Using this method, we simultaneously observed the temporal dynamics of TERT localization with apoptosis process induced by oxidative stress. Mutations in mitochondrial targeting sequence of TERT changed the cell death reaction of HeLa cells, suggesting that the sequence acts as a cell death regulator.

Chaperones, Protein Folding, and Quality Control 1

P1592/B728

Mitochondrial Dysfunction Is Signaled to the Integrated Stress Response by Oma1,mitochondrial Dysfunction Is Signaled to the Integrated Stress Response by Oma1, Dele1 and Hri.

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In mammalian cells, mitochondrial dysfunction triggers the integrated stress response (ISR), in which eIF2α phosphorylation upregulates the transcription factor ATF4. However, how mitochondrial stress is relayed to the ISR is unknown. We found that HRI is the eIF2α kinase necessary and sufficient for this relay. Using an unbiased CRISPRi screen, we identified factors upstream of HRI: OMA1, a mitochondrial stress-activated protease, and DELE1, a little-characterized protein we found to be associated with the inner mitochondrial membrane. Mitochondrial stress stimulates the OMA1-dependent cleavage of DELE1, leading to its accumulation in the cytosol, where it interacts with HRI and activates its eIF2α kinase activity. Blockade of the OMA1-DELE1-HRI pathway is beneficial during some, but not all types of mitochondrial stress, and leads to an alternative response that induces specific molecular chaperones. Therefore, this pathway is a potential therapeutic target enabling fine-tuning of the ISR for beneficial outcomes in diseases involving mitochondrial dysfunction.

P1593/B729

Therapeutic Genetic Variation Revealed in Diverse Hsp104 Homologs.

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Aberrant protein folding and aggregation underpins several fatal neurodegenerative diseases. Protein disaggregases, such as the AAA+ protein Hsp104, which directly counteract protein aggregation, represent an innovative technology to reverse these deleterious protein misfolding events. Indeed, engineered Hsp104 variants potently suppress toxicity associated with TDP-43 and αSyn in yeast and metazoa. But, these artificial disaggregases can have damaging off-target effects that might restrict their therapeutic potential. Thus, uncovering other therapeutic Hsp104s with diminished propensity for off-target effects remains a key objective. Hsp104 is conserved among all nonmetazoan eukaryotes and eubacteria, yet natural Hsp104 sequence space remains largely unexplored, raising the possibility that natural sequences exist with divergent enhanced and selective activity against neurodegenerative disease substrates. Here, we survey a cross-kingdom collection of Hsp104 homologs for their ability to suppress proteotoxicity of several proteins implicated in neurodegenerative disease. Surprisingly, nearly half of these selectively suppress TDP-43 or αSyn toxicity. Mechanistic studies and mutational analysis

suggest that these activities are traceable to genetic variation that impacts passive chaperone activity of Hsp104 homologs for select substrates. Our results suggest that natural Hsp104 homologs can have therapeutic-disaggregase activity, and that sequence variation among Hsp104 homologs may be a valuable resource for engineering therapeutic disaggregases to counter disease.

P1594/B730

Signal Peptide Processing Regulates Unfolded Protein Degradation in Mammalian Cells.

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It is estimated that in eukaryotic cells, about one third of the proteome is synthesized and matured inside the endoplasmic reticulum (ER) lumen. Efficient degradation of unfolded proteins is hence crucial to the maintenance of protein homeostasis and normal cellular functions. Although ER-associated degradation (ERAD) via proteasomes has been extensively studied and its significance underscored, knowledge about alternative protein degradation mechanisms is scarce. Using ER lumen-targeted, hard-to-fold fluorescent proteins as reporters, we discovered a signal peptide-mediated degradation mechanism of unfolded proteins. Specifically, by examining point mutations of the first amino acid residue (P1') downstream to the signal peptidase (SPase) cleavage site of the human calreticulin signal peptide, we found small, hydrophobic residues (A/G) drastically compromised the expression level as compared to naturally occurring negatively charged residues (D/E). The amount of difference in expression level depended on the protein following the signal peptide, with the more misfolding-prone ones exhibiting larger differences. Inhibition of SPase by a genetically encoded inhibitor abolished the difference, highlighting the critical role of signal peptide processing in this mechanism. On the cellular level, a type of extremely slim, recycling endosome-like organelle, together with many vesicles in the secretory pathway, emerged in cells with A/G but not D/E, implying degradation via vesicular transport. The ER-Golgi trafficking inhibitor brefeldin a and lysosomal inhibitor bafilomycin A1, but not the proteasome inhibitor MG132, suppressed the formation of such organelles in A/G as well as increased the fluorescent protein level, which corroborated a non-ERAD mechanism of unfolded protein clearance. In conclusion, we revealed an unexpected function of signal peptide processing on the sensitivity to unfolded protein degradation through secretory pathways, with negatively charged residues conferring more tolerance to unfolded proteins and thus higher protein levels in the ER.

P1595/B731

The Role of the Endoplasmic Reticulum in Quality Control of Non-Imported Mitochondrial Membrane Proteins.

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Mitochondria contain over 1,000 proteins, the vast majority of which are encoded in the nucleus and translated in the cytoplasm. These precursor proteins are imported into mitochondria by translocase complexes located on mitochondrial membranes, and this process depends on the inner mitochondrial membrane potential. As a consequence of mitochondrial dysfunction, mitochondrial protein import is impaired. Recent studies found that non-imported mitochondrial proteins cause mitochondrial precursor overaccumulation stress, which is highly toxic to the cell. To determine how cells process non-imported mitochondrial proteins as a protective protein quality control mechanism, we systematically screened the localization and abundance of yeast non-imported mitochondrial proteins by microscopy

and western blot, respectively. We identified at least five distinct fates of non-imported mitochondrial proteins. A number of these proteins localized to the endoplasmic reticulum (ER) when mitochondrial protein import was blocked by depolarization of mitochondria or genetic impairment of mitochondrial translocases. We found that the guided entry of tail-anchored proteins (GET) complex, a known post-translational ER protein import machinery, is required for the ER-targeting of non-imported mitochondrial membrane proteins. In the absence of a functional GET pathway, non-imported proteins destined for the ER are sequestered into protein aggregates that co-localize with Hsp104 and are associated with both mitochondria and the ER. Overall, these results uncover an important role for the GET complex in preventing aggregation of non-imported mitochondrial membrane proteins, and identify the ER as a major destination for non-imported mitochondrial membrane proteins.

P1596/B732

Regulation of ER Stress Resistance by Tauroursodeoxycholic Acid in Yeast.

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Accumulation of toxic misfolded secretory proteins in the endoplasmic reticulum (ER stress) is linked to several human diseases. Cells respond to ER stress through activation of signaling pathways such as the Unfolded Protein Response (UPR), which adapt the ER folding environment upon changes in misfolded protein burden. Small molecules called chemical chaperones have been shown to attenuate the UPR under stress conditions and improve folding and trafficking of specific mutant proteins. Here, we aimed to identify the effects of a chemical chaperone, the bile acid tauroursodeoxycholic acid (TUDCA), in yeast. Despite promising results in multiple models of protein folding diseases, TUDCA's mechanism of action remains unclear. We found that TUDCA significantly improves growth of yeast subjected to ER stress caused by tunicamycin (Tm). Interestingly, the ability of TUDCA to rescue growth in presence of Tm was independent of the UPR. Moreover, TUDCA could not attenuate UPR activation associated with deletion of genes involved in protein quality control, suggesting that it may act by increasing cell resistance to pharmacological stressors. Using genome-wide screens, we have linked the ability of TUDCA to rescue Tm-induced stress to the regulation of yeast cell wall integrity. Cells lacking the MAP kinase Slt2, a protein essential to signal transduction through the cell wall integrity (CWI) pathway, were hypersensitive to TUDCA. This suggests that TUDCA treatment results in cell wall remodeling, thereby requiring CWI signaling. Likewise, using quenching assays, we were able to demonstrate that TUDCA decreases cell wall porosity and increases cell resistance to the cell wall-targeting agent and ER stress inducer, caspofungin. Thus, we characterized a novel mechanism of action of TUDCA in increasing ER stress resistance by regulating cell wall integrity and presumably affecting drug uptake. Supported by NSERC.

P1597/B733

Hsp104/70/40 Disaggregation System Disperses Heat-induced Poly(a)-binding Protein Aggregates.

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All cells produce an adaptive response to environmental stresses, including heat. Two highly conserved features of the cellular heat shock response are 1) an increased production of heat shock proteins, many of which are molecular chaperones, and 2) aggregation of protein and mRNA molecules into stress

granules (SGs). During the recovery period, a set of molecular chaperones (Hsp104/70/40) disperses the constituent SG proteins back to the cytosol. We previously demonstrated that poly(A)-binding protein (Pab1 in budding yeast; PABPC1 in human), a classic SG marker, demixes in vitro upon exposure to physiological stress conditions. Pab1 demixing involves phase separation and gelation without global unfolding. Although the Hsp104 disaggregation system has been shown to be important for the timely dispersal of SGs in vivo, no direct demonstration of Hsp104/70/40-mediated dispersal of endogenous, stress-induced aggregates has been done. Here, using Pab1 as model substrate, we demonstrate that Hsp104 disaggregation system can disperse endogenous stress-induced aggregates. We introduce a fluorescence anisotropy assay for kinetic monitoring of the conversion of inert Pab1 aggregates into soluble RNA-binding Pab1 monomers. Pab1 dispersal requires co-chaperone Sis1 but not Ydj1. Unlike in the case of misfolded substrates, no synergy between Sis1 and Ydj1 is observed with Pab1. The high resolution of our assay and the substoichiometric concentration of molecular chaperones required for Pab1 dispersal make our system a useful tool for studying molecular chaperones in the context of disaggregating RNA-binding protein aggregates. We intend to exploit our system to address important questions as to the mechanisms by which the disaggregation system recognizes Pab1 and the molecular features, e.g. phosphorylation, which control the efficiency of molecular dispersal of distinct stress-induced structures.

P1598/B734

Deciphering the Role of ER Stress in ALS Pathogenesis.

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There is considerable evidence showing chronic induction of ER stress is associated with many neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). However, direct evidence that ER stress is responsible for causing disease is more difficult to ascertain. This is because of the difficulty in separating the effects of ER stress from the gain or loss-of-function of the instigating molecule responsible for triggering the ER stress. For example, retention and build-up of a misfolded protein in the ER by the protein quality control systems could trigger ER stress but could also produce loss-of-function effects from retention of the molecule. To overcome this problem, we generated transgenic mice (Tg) expressing CD3δ tagged with Dendra2 in neurons. CD3δ is one of several subunits that forms the T-cell antigen receptor complex. It is an ideal substrate for studying ER-associated degradation (ERAD) because expression of the subunit without its cognate partners is recognized by the ER quality control systems as being an orphan protein and is therefore targeted by ERAD. Thus, ectopic expression of CD3δ-Dendra2 in non-T cells, such as in neurons, should result in elimination of the protein by ERAD. However, we predicted that the prolonged expression of CD3δ-Dendra2 in neurons would trigger ER stress from a build-up of the protein as the quality control systems in organisms wane during aging. A transgenic mouse line expressing CD3δ-Dendra2 was generated and characterized. The line was also crossed with a P497S UBQLN2 mouse model of ALS, which we previously showed recapitulated central features of the human disease. We predicted that double transgenic mice from this cross would not only develop disease earlier, but disease would be exacerbated because of strong induction of ER stress seen in affected P497S animals. The strong induction of ER stress is P497S animals is consistent with our previous studies showing UBQLN proteins function in ERAD. Quantification of the number of motor neurons (MN) in spinal cord sections of single transgenic CD3δ-Dendra2 expressing mice revealed an age-dependent decline, which correlated with induction of ER stress markers. Furthermore, mice double transgenic for the reporter and the P497S UBQLN2 transgene displayed increased MN loss compared to

age-matched single Tg mice carrying each of the transgenes. Our results suggest that chronic induction of ER stress may be sufficient to trigger motor neuron disease. The CD3δ-Dendra2 transgenic mouse line appears to be a useful tool for interrogating the effects of ER stress in neuron function and neurodegeneration.

P1599/B735

Conformational Heterogeneity in the α-Synuclein Aggregation Leads to the Formation of Diverse Strains with Distinct Prion-like Behavior.

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α-Synuclein (α-Syn), a neuronal protein, is misfolded and aggregated in several neurodegenerative diseases, termed as α-Synucleinopathies. While sharing the same pathological protein, synucleinopathies demonstrate distinct clinical and pathological phenotypes. The coalition of protein aggregates made up of the same precursor protein but with multiple pathological phenotypes occurring due to the presence of different conformational strains has been demonstrated for prion diseases. However, the factors that are responsible for the formation of prion-like strains in synucleinopathies is still obscure. α-Syn forms heterogeneous high-molecular-weight assemblies through a series of conformational changes as well as intermolecular interactions. Variation in incubation conditions and/or in the presence of modulator/cellular factors, α-Syn may populate into numerous conformational states to form partially folded intermediates and protofibrils. Owing to the heterogeneity in the protein aggregation and cellular systems, individual species involved in the aggregation pathway may deviate and populate into different types of structural assemblies, in *vivo*. Here, we strategize to isolate the intermediate species formed during the α-Syn aggregation pathway and study their ability to form different fibrillar strains. Using interdisciplinary tools, we comprehensively characterized two α-Syn variants (named as PMF and HMF) formed in the process of aggregation. These variants displayed structural differences as probed by solid-state NMR and Hydrogen-deuterium exchange mass spectrometry as well as exhibited significant functional differences in terms of cytotoxicity and prion-like transmission behavior. We demonstrated that HMF are more resistant to protease digestion, and these fibrils are also protected against Hydrogen-deuterium exchange (HDX). Whereas, PMF are readily digested with proteinase-K and get labeled upon HDX displaying it to be a less protected fibril type. In the cellular context, these strains exhibited distinct internalization and transmission ability. Although PMF strain showed comparatively less internalization and cell-to-cell transmission compared to the HMF, it presented relatively high seeding ability both in *vitro* and in *cells*. Therefore, studying these different structural assemblies will be a step closer to simulate the in *vivo* conditions delineating the differences in the pathologies and designing conformation-based inhibitors of α-Syn.

P1600/B736

Analyzing Premature Substrate Release from ClpXP with Single Molecule Microscopy.

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AAA+ proteases, such as proteasomes, include a subcomplex that engages, translocates and (if required) unfolds tagged substrates, before delivering them to an associated protease particle. Occasionally, substrates can be prematurely released, resulting in partially degraded products that may have various physiological impacts. A potential cause of partial degradation is the presence of domains that reduce

the grip that the AAA+ proteases has on the substrate. This is manifested in the low rate of degradation for proteins with glycine-alanine (GA) repeats found in Epstein-Barr viruses, indicating that the lack of bulky side-chains in the amino acid sequence makes the substrate more slippery. However, it is not resolved whether this effect of GA repeats results from an increase in the likelihood of substrate dissociation, or a prolongation of the time needed to unfold engaged substrates, or a combination of both possibilities. In this study, we dissected the problem by monitoring the dissociation of protein substrates from the AAA+ protease ClpXP at single molecule resolution using Total-Internal-Reflection Fluorescence (TIRF) microscopy under a quasi-steady-state condition. We designed a protein substrate containing a domain encoding the *E. coli* dihydrofolate reductase (ecDFHR), which is a hard-to-unfold protein that becomes fully resistant to unfolding upon binding methotrexate (MTX). By placing a GA repeat at the C-terminal non-structured region of the ecDHFR substrate, we forced ClpXP to stall at or near the GA repeat. Comparing the degradation rates of the ecDHFR substrates with, or without, the GA repeats in an ensemble assay in a defined solution, we confirmed the observation that the presence of GA repeats leads to a lower rate of degradation by ClpXP. After examining dwell-time of MTX-bound substrate with TIRF microscopy, we found that GA repeats had little effect on the rate of substrate dissociation. We also studied the interaction between ClpXP and ecDHFR substrates with Surface Plasmon Resonance (SPR) to probe the equilibrium of substrate binding and dissociation. Together, the results suggest that GA repeats impede protein degradation without making the substrates more likely to dissociate from the AAA+ proteases, implying that there is a difference in the mechanisms of interaction by which ClpXP unfolds and retains the substrate.

P1601/B737

Functional Cooperativity between the Trigger Factor Chaperone and the ClpXP Proteolytic Complex.

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A functional association was uncovered between the ribosome-associated trigger factor (TF) chaperone and the ClpXP degradation complex in bacteria. Bioinformatic analyses demonstrated conservation of the close proximity of *tig*, gene coding for TF, and genes coding for ClpXP suggesting a functional association. Effect of TF on ClpXP-dependent degradation varied based on the nature of substrate. While degradation of some substrates was slowed down or unaffected by TF, surprisingly, TF increased the degradation rate for a third class of substrates. These include λ phage replication protein λO, master regulator of stationary phase RpoS, and SsrA-tagged proteins. Globally, TF enhances the degradation of about 2% of newly synthesized proteins. TF was found to interact through multiple sites with ClpX in a highly dynamic fashion to promote protein degradation. This chaperone-protease cooperation constitutes a unique and likely ancestral aspect of cellular protein homeostasis in which TF acts as an adaptor for ClpXP.

P1602/B738

Phosphorylation-dependent Proteolysis Perturbs GFAP Proteostasis, Implicating Caspase-6 in Early Onset Alexander Disease.

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Glial fibrillary acidic protein (GFAP) is the major component of the intermediate filament cytoskeleton in mature astrocytes. *GFAP* mutations cause the rare and fatal leukodystrophy Alexander Disease (AxD), via a toxic gain-of-function mechanism involving pathologic aggregation of mutant GFAP within Rosenthal Fibers (RFs). Our major objective is to understand the causes and consequences of GFAP aggregation and RF formation in AxD. Although there are over 70 different GFAP missense mutations known to cause AxD, the underlying mechanism linking different mutations to disease-relevant phenotypes remains unknown. We used AxD patient brain tissue and induced pluripotent stem cell (iPSC)-derived astrocytes to investigate the hypothesis that AxD-causing mutations perturb key post-translational modifications on GFAP. Phospho-proteomic analysis was performed on GFAP extracted from post-mortem tissues from AxD patients (representing 10 different mutations) and control unaffected subjects. The proteomic results were confirmed by immunoblot with phospho-specific antibodies. Mechanistic studies were undertaken in human AxD iPSC-astrocytes and corresponding isogenic controls that were generated via CRISPR/Cas9 gene editing. Our results reveal abundant, selective phosphorylation of GFAP on Ser13 in patients who died very young (N=8; median age at death=1.7 years; range 0.5-14 years), independently of the mutation they carried. We did not observe significant pSer13-GFAP in control subjects or in AxD patients who survived longer (N=5; median age at death=38 years; range 27-50 years). Human AxD iPSC-astrocytes accumulated pSer13-GFAP in cytoplasmic aggregates within deep nuclear invaginations, resembling the hallmark RFs observed in *vivo*. In contrast, isogenic control iPSC-astrocytes lacked pSer13-containing GFAP aggregates. While WT and non-phosphorylatable S13A GFAP variants were capable of assembling into filaments, the phospho-mimic S13D GFAP variant formed large perinuclear aggregates. Furthermore, Ser13 phosphorylation facilitated GFAP cleavage by caspase-6, which was only expressed in the brain tissue from young AxD patients. Consequently, in brain tissue from young AxD patients there was abundance of cleaved GFAP, which was not seen in old AxD patients or in normal subjects (young and old). In conclusion, our study reveals how missense mutations, affecting discrete domains on the GFAP molecule, converge upon a common mechanism to compromise GFAP proteostasis in AxD. Our findings provide a basis for exploring potential PTM-based diagnostic and therapeutic strategies in AxD and potentially other diseases characterized by defective intermediate filament proteostasis.

P1603/B739

Giv Interacts with and Promotes Cell Surface Localization of Grp78 during ER Stress.

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Cancer cells are known to evade endoplasmic reticulum (ER) stress induced death and one of the key factors that helps in survival is cell surface localization of the chaperone, glucose regulated protein 78 kDa (GRP78), from where it aids in promotion of cytoprotective signaling. However, the mechanism by which GRP78 localizes to the plasma membrane remains poorly understood. Here, we identify Gα-Interacting Vesicle Associated Protein (GIV) - an enhancer of the pro-survival signaling during ER stress - as a binding partner of GRP78. We show that GIV and GRP78 interact in an ER-stress dependent manner. This direct interaction occurs through binding between the substrate-binding domain of GRP78 and the carboxyl-terminal region of GIV. GIV promotes the ER stress dependent localization of GRP78 to the plasma membrane as determined by cell surface biotinylation experiments. Finally, Kaplan-Meier analysis of disease-free survival in breast cancer patients shows poor prognosis for patients with high expression of both GIV and GRP78. Taken together, our work suggests a crucial role for GIV in facilitating

localization of GRP78 to the plasma membrane, from where these two proteins can promote cell survival signaling.

P1604/B740

Cellular Secretion and Cytotoxicity of Transthyretin Mutant Proteins Underlie Late Onset Amyloidosis and Neurodegeneration.

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Transthyretin amyloidosis (ATTR) is a progressive life-threatening disease characterized by the deposition of transthyretin (TTR) amyloid fibrils in the body's organs and tissues, causing familial amyloid polyneuropathy (ATTR-FAP), cardiomyopathy (ATTR-FAC), or oculoleptomeningeal amyloidosis (OLMA). Several pathogenic variants have been shown to destabilize TTR tetramers, leading to aggregation of misfolded TTR fibrils. However, factors that underlie the differential age of disease onset amongst amyloidogenic TTR variants remain elusive. To understand these phenotypic differences, we generated TTR mutants with different disease onsets and study their amyloidogenic properties in cultured cells and Drosophila melanogaster. We found similar cellular secretory pattern in both HEK and HepG2 cells for wild-type (WT) TTR to those of the late-onset mutant (Ala97Ser), stable mutant (Thr119Met), early-onset mutant (Val30Met), but not unstable mutant (Asp18Gly). Cytotoxicity assays revealed their toxicities in the order of Val30Met>Ala97Ser>WT>Thr119Met in IMR-32 neuroblastoma cells. Surprisingly, while early onset amyloidogenic TTR monomers are retained by the endoplasmic reticulum quality control (ERQC), late-onset amyloidogenic TTR monomers can be secreted extracellularly. Interestingly, Ala97Ser TTR overexpression in *Drosophila* causes late-onset neurodegeneration but aggressively shortened lifespan, recapitulating human disease progression. Our study demonstrates that escape of TTR monomers from the ERQC may underlie late-onset amyloidogenesis in patients and suggests potential treatment strategies targeting ERQC for mitigating late-onset ATTR.

P1605/B741

Unexpected Control of Nucleation by Protein Translation Rate.

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Protein translation is the most energy-consuming process in the cell. Thus, translation and protein quality control must be tightly linked. Proteins are believed to be most susceptible to aggregation during or shortly after translation, before they have reached the fully folded native state. We put that notion to test by evaluating nucleation-limited, or "prion-like" aggregation under different rates of translation in the cell. We included prions of diverse structure, ranging from intrinsically disordered amyloid-forming proteins (IDPs), to well-folded death domains that polymerize in the native state. Remarkably, death domains retain their native fold even in the prion form, so any misfolding may only perturb their attaining the prion form. The expectation is therefore that translation rate will hamper death domain nucleation. To probe this, we deployed a quantitative reporter of nucleation-limited protein self-assembly in living cells (termed Distributed Amphifluoric FRET or DAmFRET). To determine if these

effects occur directly at the level of the translating prion protein-encoding transcript itself, we inserted an out-of-frame start codon immediately upstream of the ORF to decelerate translation initiation specifically of our prion protein of interest. Strikingly, this manipulation dramatically reduced nucleation for all proteins tested, regardless of their structure, and independently of changes in their concentration. Further experimentation revealed that the proteins remained fully competent for polymerization when provided with a pre-existing template. Hence, translation rate specifically influences the kinetic barrier to nucleation. Overall, our observations are true in both yeast and mammalian cells alike. We are exploring microscopy-based tools to visualize how translation governs the physical basis of protein phase behavior. Given the generality of the effect of translation on the nucleation of a wide range of proteins, the mechanism quite likely involves the increased local concentration of newly translated polypeptides occurring at the polysome.

P1606/B742

Overexpression of UBQLN1 Alleviates Motor Neuron Loss and Neuropathology in the P497S UBQLN2 Mouse Model of ALS/FTD.

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Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disease characterized by progressive loss of upper and lower motor neurons of the central nervous system. Median survival after diagnosis is typically 3 to 5 years, and there is no effective treatment to prevent or halt the disease. Major insight into ALS has emerged through discovery that mutation in more than 30 different genes cause ALS. Interestingly, mutations in genes involved in protein quality control are linked to ALS more than any other functional group. Mutations in one such factor, UBQLN2, have been identified as a genetic cause of ALS and frontotemporal dementia (FTD). UBQLN2 belongs to a family of four homologous proteins found in humans that function in protein quality control. The proteins share the same structural organization composed of N- and C-terminal ubiquitin-like (UBL) and ubiquitin-associated (UBA) domains and a central domain containing multiple STI1 domains, that bind to poly-ubiquitin chains and the proteasome respectively. These structural features are consistent with the diverse functional properties played by the proteins as shuttle factors in proteasomal degradation, facilitators of autophagy and in assisting in protein refolding. Most UBQLN2 mutations map to a PXXP domain that is only present in UBQLN2 and not in the other UBQLN family members. We previously showed that overexpression of UBQLN1, which shares high homology to UBQLN2, can rescue toxicity in cell and animal models of Huntington's disease. Accordingly, we speculated that overexpression of UBQLN1 may counteract and alleviate the pathogenic effects of UBQLN2 ALS/FTD mutations. To test this idea, we crossed transgenic (Tg) mouse lines expressing human UBQLN1 with the P497S UBQLN2 mouse model of ALS/FTD. The P497S mouse model displays age-dependent motor neuron disease, cognitive deficits and neuropathologic alterations (build-up of ubiquitin and p62-positive inclusions and TDP-43 proteinopathy), recapitulating central feature of the human disease. All four genotypes from the resulting cross were characterized pathologically. The characterizations revealed that UBQLN1:P497S double-Tg mice had dramatic reduction of UBQLN2 inclusions in the brain and spinal cord, diminished loss of neurons in the brain and of motor neurons in the spinal cord, as well as alleviation of ER stress and of TDP-43 mislocalization. Taken together these results demonstrate that overexpression of UBQLN1 in P497S mice alleviates most of the pathologic signs of disease caused by the UBQLN2 mutation. Therefore, strategies to increase UBQLN1 expression may provide an attractive therapeutic opportunity to treat ALS/FTD caused by UBQLN2 mutations.

P1607/B743

The Lon Protease Autodegrades in *Caulobacter Crescentus*.

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Lon is a highly conserved AAA+ protease found in prokaryotic and eukaryotic organisms; it relies on the hydrolysis of ATP in order to remove damaged or misfolded proteins from the cell as a part of the cells natural stress response. In *Caulobacter crescentus*, Lon is also capable of regulating the concentrations of key regulatory proteins responsible for a variety of cell functions like growth and motility, regulated proteolysis is a vital function of healthy bacterial growth. So, when this carefully balanced system is disrupted it often results in reduced viability. Because protein turnover is irreversible and costly, cells must carefully regulate their protease activity, Lon is controlled by transcriptionally upregulating the *lon* gene in the presence of high stress or at decisive cell cycle checkpoints. The question remains however, after the cell has responded to the stress and survives, what happens to the surplus of Lon protease in the cell. Here we show that Lon itself is degraded and that it plays an important role in its own post-transcriptional regulation. Using mutants deficient only in catalytic activity, we find that Lon targets itself for degradation. In preliminary work, we find that stabilizing catalytically active Lon appears to be toxic for *Caulobacter*, suggesting that the turnover of Lon is needed during normal growth. By studying how Lon is under its own proteolytic control, we hope to further our understanding in Lon's capability to respond to stress conditions and achieve cell homeostasis.

P1608/B744

Interaction of the Yeast Smyd Methyltransferase Set6 with the Gim Complex/prefoldin Links Its Function to the Regulation of Proteostasis.

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The SMYD family of lysine methyltransferases are key developmental regulators, particularly implicated in muscle differentiation, and known to promote pathogenesis when deregulated, including being overexpressed in numerous types of cancer. The enzymes contain a split SET (Su(var)3-9, Enhancer of zeste, Trithorax) domain that catalyzes methylation on target substrates, and a MYND (Myeloid translocation protein, Nervy, Deaf) domain, which is a zinc finger motif that promotes both protein-protein and protein-nucleic acid interactions. In mammalian systems, SMYD enzymes are known to catalyze both histone and non-histone protein methylation, although the extent of their methylation of the proteome and the signaling pathways to which they respond are not clear in many cases. In genetic model systems, the SMYD family of enzymes are highly-conserved but their characterization is very limited. Here, we sought to define the biological function of the budding yeast enzyme Set6, a SMYD family member without defined function or known substrates. We identified a physical and genetic link between Set6 and the GimC/prefoldin complex, a protein chaperone that receives nascent chains at the ribosome and transfers them to the chaperonin CCT/TRiC. The loss of Set6 suppresses many of the defects associated with loss of members of the GimC/prefoldin complex, suggesting that they function in the same pathway. Additional genetic data links Set6 to translational control, indicating that Set6 may play a regulatory role in translation that antagonizes GimC/prefoldin function. Further, biochemical studies show that Set6 is an active methyltransferase that targets multiple substrates within the cell.

Overall, our data suggest that Set6 acts together with GimC/prefoldin to regulate protein abundance and quality control, implicating the SMYD lysine methyltransferase family in the maintenance of proteostasis and defining a new biological function for this previously-uncharacterized enzyme.

P1609/B745

Cytoplasmic Phosphorylation of FUS's Prion-like Domain as a Mechanism to Regulate Liquid-liquid Phase Separation.

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Fused in Sarcoma (FUS) is a ubiquitously expressed predominantly nuclear protein with many RNA/DNA metabolism-related functions. FUS contains a prion-like domain that facilitates its liquid-liquid phase separation into functional granule structures. Chronic cytoplasmic persistence of this phase-separated state is implicated in diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). In cell models a sub-population of FUS is localized within cytoplasmic stress granules following osmotic and oxidative stress. Likewise, disease-associated mutant protein, FUS(R495X), forms liquid-state cytoplasmic inclusions. We previously showed following DNA damage, PIKK kinases phosphorylate consensus sites within FUS's prion-like domain altering liquid-phase transitions. Here, we show that transient phosphorylation of non-PIKK consensus sites occurs following non-DNA damaging stress. Within cytoplasmic inclusions we observe FUS's prion-like domain to be phosphorylated at PIKK and non-PIKK sites. These phosphorylation events are not PIKK-kinase dependent, indicating additional kinases are modifying FUS's prion-like domain. Phosphomimetic substitution within the prion-like domain of mutant FUS changes the persistence of cytoplasmic liquid-state granules. Our findings suggest prion-like domain phosphorylation is a mechanism to regulate FUS's liquid-liquid phase separation. Modulation of prion-like domain phosphorylation may offer therapeutic strategies for ALS and FTD.

P1610/B746

Selective, Stress-enhanced ER-export of Misfolded Transmembrane Proteins.

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To avoid accumulation and aggregation of misfolded secretory pathway proteins, cells utilize protein quality control (PQC) pathways to recognize and refold or destroy misfolded secretory pathway proteins. The best characterized clearance pathways for misfolded secretory pathway proteins are ER associated degradation (ERAD) or autophagy, both of which involve the retention of misfolded proteins in the ER prior to degradation. While probing PQC systems that handle diverse, disease-associated misfolded GPI-anchored proteins (GPI-APs), including mutants of prion protein, we discovered an ER-stress enhanced PQC pathway that we named RESET. RESET is the major pathway used by cells to clear misfolded GPI-APs out of the ER for constitutive or ER stress-enhanced turnover. Instead of being retained in the ER for degradation, during RESET, misfolded proteins are released by the ER-resident chaperone calnexin and bound by the p24-family member Tmp21 for export to the Golgi. Tmp21 is required for ER-export to the Golgi. The misfolded proteins subsequently transit the cell surface en route to lysosomes where they are degraded. We now find that a sub-population of select misfolded transmembrane proteins, including hypercholesterolemia mutants of LDL receptor (LDLR) C184Y and C358Y and cysteine mutants of amyloid precursor protein (APP), are also released by calnexin for the Tmp21-mediated RESET pathway. siRNA knockdown of Tmp21 studies demonstrate that Tmp21 is

required for ER-export of LDLR C184Y. In contrast, the well-characterized transmembrane ERAD substrate, CD3δ, appears to be exclusively handled by ERAD and does not appear to associate with Tmp21. As with GPI-APs, after undergoing RESET, the APP and LDLR mutants continue through the Golgi to the cell surface where they are rapidly internalized to lysosomes. Next we tested for the consequence of the transient cell-surface exposure of these RESET substrates. We discovered that the fraction of LDLR mutants that undergoes RESET are capable of LDL uptake. Imaging studies revealed that LDLR C184Y binds fluorescently labeled LDL ligands at the cell surface and internalizes the ligands into lysosomes. In the case of APP mutants, triggering RESET with ER-stress spikes the release of amyloid beta from cells as determined by ELISA. Thus, the fraction of misfolded transmembrane proteins that undergoes RESET for secretion to the cell surface may have potentially beneficial or detrimental physiological consequences, depending on the function of the RESET substrate. Additionally, our discovery that a fraction of APP mutants associate with Tmp21 for RESET may provide a new lead to determine the mechanistic basis for the genetic link between Tmp21 and Alzheimer's disease that was identified by other labs.

Systems and Synthetic Cell Biology

P1611/B748

Phase Separation of a Structured Protein Domain.

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Liquid-liquid phase separation has been proposed to drive assembly of many functionally distinct cellular compartments not enclosed within lipid membranes. It is likely that material properties of these compartments regulate their functional output. Research from many labs have shown how both compartment assembly and material properties depend on scaffold proteins that are intrinsically disordered. However, the contribution of structured protein domains in this process remain unclear. To address this, we investigated the phase separation properties of PGL-3, one of the scaffold proteins that drive assembly of the canonical liquid-like 'P granule' compartment in *C. elegans*. We found that in the case of PGL-3, both compartment assembly and material properties depend predominantly on a structured protein domain in contrast to intrinsically disordered regions. The N-terminal region accounting for 65% of the PGL-3 protein (PGL-3_N) is alpha-helical. On the other hand, the C-terminus (PGL-3_C) accounting for the other 35% of the protein is unstructured. While the N-terminal fragment PGL-3_N phase separates identical to full-length PGL-3 in vitro, the C-terminal fragment PGL-3_C is neither necessary nor sufficient for phase separation. We explored the molecular mechanism of how the N-terminal fragment PGL-3_N regulates material properties in PGL-3 condensates. Interestingly, we identified a 25 amino acid region within PGL-3_N (PGL-3_liq), accounting for just 4% of the protein, that maintains 'liquidity' in PGL-3 condensates. PGL-3 constructs lacking the PGL-3_liq sequence phase separate into condensates that harden over time from a liquid-like state to a solid-like state. We found that the activity of PGL-3_liq in maintaining liquidity is sequence-specific, since a polypeptide of identical length and amino acid composition but with the sequence of amino acid residues scrambled could not substitute for the role of PGL-3_liq. I will discuss in detail our findings on how PGL-3_liq regulates material properties in PGL-3 condensates. Our work demonstrates the importance of structured protein domains in liquid-liquid phase separation and sets the stage for investigations into how structured

protein domains cooperate with intrinsically disordered protein regions to assemble cellular liquid-like compartments.

P1612/B749

Critical Role of Rhythmic Poly(a) Tail Regulation in Circadian Gene Expression.

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Circadian gene expression has been well understood at the transcriptional level for decades. Core clock genes with their protein products form negative feedback loops essential for temporal oscillation. However, recent genome-wide studies highlighted the role of post-transcriptional regulation in circadian gene expression. Particularly, rhythmicity of poly(A) tail lengths are found to strongly correlate with the rhythmicity in protein expression in mouse liver. Poly(A) tail is generally thought to control mRNA decay and translation activity. To understand the specific role of rhythmic poly(A) tail regulation in circadian gene expression, we constructed a simplistic model that depicts rhythmic control imposed upon basic mRNA expression processes, including transcription, deadenylation, polyadenylation and degradation. Our results revealed rhythmicity in deadenylation as the strongest contributor to the rhythmicity in poly(A) tail length, and the abundance of long-tailed mRNA. Our model further demonstrated that the rhythmic patterns in the expression of several deadenylase subunits could cluster the peak phases of poly(A) tail length and long-tailed mRNA abundance, respectively, into three distinct time windows around the clock, regardless of the rhythmic features in transcription, polyadenylation and degradation. This behavior could allow rhythmicity in deadenylase expression to be a strong organizer for circadian gene expression across the genome. Last, our model suggested factors that could contribute to the experimentally observed rhythmicity in poly(A) tail length and total mRNA abundance.

P1613/B750

Identification of Gene Regulatory Networks for Robust Tissue-scale Spatial Pattern Formation during Morphogenesis.

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Proper tissue and organ function are dictated, in part, by the correct spatial organization and interaction of multiple cell populations. During embryonic development, this coordinated spatial and temporal organization is frequently achieved by having sender-receiver type signaling systems between different cell populations. In these systems, specific morphogens are secreted by sender cells and receiver cells then sense the morphogen gradient using a network of transcriptional activators/repressors called the intracellular gene regulatory network (GRN) to determine their function. This sensing is influenced by stochastic noise appearing at the cellular and multi-cellular tissue scale. Cellular noise arises from gene expression noise and diffusion, degradation, and secretion of morphogens whereas spatio-temporal fluctuations in growth factors, cell movement, cell proliferation and cell death drive noise at the tissue scale. We developed a computational tool to infer the minimal structure and parameters of GRN and intercellular interactions within interacting cell types to explain experimentally observed spatial gene expression patterns. To validate the computational tool, a bacterial system is used with a sender cell population that secretes a morphogen that diffuses through the medium into a receiver cell population to activate receiver gene expression. Reporter genes are used to image and quantify gene expression intensities in both cell populations. Stochasticity can be disadvantageous to receiver cell fate patterning

as the cells must transduce the morphogen gradient and reliably lock into a particular cell fate and prevent random switching. This feature is encoded in the interplay of the GRN and the intercellular morphogen exchange. Hence, we develop and simulate a stochastic reaction-diffusion model in silico and rank order the inferred networks based on the robustness of their generated patterns against stochastic gene expression and random spatial distribution of cell-types. This tool provides insights into optimizing the inter- and intracellular networks for reducing the effect of stochasticity on pattern formation at the tissue-scale needed for proper morphogenesis.

P1614/B751

Ph-triggered Coacervate Formation Activates Enzyme Reactions.

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The field of liquid-liquid phase separation (LLPS) in biology has attracted much attention over the last decade and bimolecular condensates have now been identified in a wide range of eukaryotic organisms. Despite this, the precise function of membrane-free compartments within biological systems has not been entirely elucidated. As biology is innately complex, it is often challenging to determine the function of condensates within the myriad of biochemical processes. On the other hand, in *vitro* assays provide a simplified bottom-up approach that allow for the controllable testing of condensate function. We have therefore developed a method for the *in-situ* formation of coacervates (a form of condensates) within giant unilamellar vesicles (GUVs) to enable the controllable testing of the role of phase separation in regulating biochemical reactions. Coacervation, a specific type of LLPS driven by the electrostatic attraction of oppositely charged polymers, has been implicated as one of the driving forces for condensate formation. Here, we exploit the intrinsic pKa of coacervate-forming polymers polylysine and either ATP or carboxymethyl dextran to reversibly drive coacervate formation within lipid vesicles with pH changes. We show that the *in-situ* coacervate formation activates dormant reactions in solution, by the up-concentration of the reactants. Our results, demonstrate a clear functional role of condensates and show that minimal and dynamic in *vitro* models can offer fundamental insights into our understanding of membrane-free condensates in biology.

P1615/B752

Differentiated Embryonic Chondrocyte Gene 1 (*Dec1)* Deficiency Inhibits Tac-induced Fibrosis in Cardiac Hypertrophy.

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Basic helix- loop-helix (BHLH) transcription factor *Dec1* (differentiated embryonic chondrocyte gene 1) is associated with tumor progression, inflammation and metabolism. However, the effect of *Dec1* on cardiac function and cardiac fibrosis is still not known. We performed TAC (transverse aortic constriction) in both of wild type (WT) and *Dec1* knock out (KO) mice. After TAC operation, all mice were assessed cardiac function by echocardiography as well as heart tissues were collected to analysis fibrosis and inflammation by immunohistochemistry and real time PCR. We found that fractional shortening (FS%) did not attenuate and *BNP* expression was lower in TAC-operated *Dec1* KO mice whereas TAC induced more cardiac hypertrophy in *Dec1* KO mice than WT mice, exhibiting an increase in heart weight/body weight ratio and thickness of left ventricle post wall. In addition, TAC-induced cardiac

fibrosis was decreased in *Dec1* KO mice, involving αSMA, TGFβ1 and TNFα expression. These results indicate that *Dec1* deficiency prevents cardiac dysfunction and inhibits cardiac fibrosis, suggesting an important role of *Dec1* in cardiac fibrosis.

P1616/B753

Application of RNA Interference for Development of Capric Acid-rich *Camelina Sativa* Germplasm.

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Camelina sativa has been shown to be a viable feedstock for biodiesel and bio-based jet fuel. Such jet-fuel generates up to 75% lower greenhouse gas emissions relative to petroleum-derived jet fuel during production and use as a commercial aviation fuel. Therefore we decided to enhance the value of camelina seed oil for bio-jet fuel by increasing its content of medium-chain fatty acids (C10:0, C12:0, C14:0) through application of RNA interference. The obtained results demonstrated the feasibility of engineering *C. sativa*, including a Ukraine-adapted variety with high yield level, for the production of oils with up to 30% of medium-chain fatty acids. Such oils are more readily convertible to bio-jet fuel than conventional camelina oil that is rich in C16-C22 fatty acids. Levels of medium chain fatty acids were further increased in camelina seed oil by silencing expression of genes (AAE15/16) for enzymes that limit accumulation of these fatty acids. Obtained results on two successfully breeded T_3 lines of engineered camelina germplasm showed increase of C10:0 content in seed oil up to 13% and 14%. The *C. sativa* varieties generated by this research will promote enhanced profitability for dryland farmers and an optimized, low carbon emission feedstock for the aviation fuel industry.

P1617/B754

Major Royal Jelly Protein 1 Increases Cell Number and Recombinant Protein Production in Cho Cells.

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Major Royal Jelly Protein 1 increases cell number and recombinant protein production in CHO cells A. Robles-Zamora[1], M. A. Torres-Acosta[1,2], J.M. Aguilar-Yáñez[1], M.E.G. Brunck[1] [1]Centro de Biotecnología FEMSA, Tecnológico de Monterrey, Monterrey, Mexico [2] Advanced Centre for Biochemical Engineering, University College London, London, UK CHO cells are commonly used to produce industry-relevant recombinant proteins that require post-translational modifications. A common research goal is to find new strategies to increase production yield, which augments product availability while reducing cost of production. Major Royal Jelly Protein 1 (MRJP1) is the main protein component of Royal Jelly, the only source of nutrition in the beehive for queen bees. Royal Jelly consumption leads to increased lifespan and body size amongst other features in queen bees. Here we report the supplementation of culture media with recombinant MRJP1 protein in wild-type cells (CHO-S) and a CHO system engineered to produce galsulfase (Gal-CHO), a drug used in the management of Fabry disease. MRJP1-supplemented medium yields significantly more CHO-S and Gal-CHO cells, 39.61% (SD±11.52%) and 36.21% (SD±11%), respectively, than vehicle-supplemented medium after 4 days in culture. In addition, specific galsulfase production was significantly increased in Gal-CHO cell in MRJP1-supplemented cultures at day 4 (54.92% SD± 20.52%, t-student test, $p<0.001$). We are currently investigating the molecular mechanisms involved in the observed phenotype. We suggest MRJP1 supplementation may be a commercially suitable alternative to expensive growth factors or complex boosts mixtures to increase industry-relevant recombinant protein production in CHO recombinant systems.

P1618/B755

Honey Proteomics: Environmental Biomonitor of the Ecosystem Health and Potential Diagnostic Biomarkers for Honeybee Disease.

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Honeybees play an important environmental role as pollinators and biodiversity promoters in both farm-growth and non-farm-growth plants. Every year, 15% of the total colonies in the USA are lost due to a combination of environmental factors including pathogens, exposure to pesticides, and climate change. In order to reduce honeybee mortality, it is important to identify the precise biological factors such as bacteria, viruses, fungi, and pathogens which cause increased mortality among honeybee populations. In this work, we focused on identifying honey proteins and peptides to use them as an environmental biomonitor and a tool for the diagnosis of honeybee disease. Because of the low concentration of protein in honey (0.1-0.5%) and the high concentration of sugars, identifying low abundance peptides and proteins causes unresolved challenges. To overcome this problem, we used multifunctional core-shell nanoparticles, which are a concentration method based on an affinity bait covalently bound to a polymer nanoparticle. When applied to a protein solution, the nanoparticles rapidly capture, concentrate and preserve solution-phase analytes, which can be then measured with standard analytical methods. In this study, we analyzed 15 honey samples collected in Virginia, Maryland, and Argentina with tandem mass spectrometry using a Thermo Fusion Orbitrap mass spectrometer and proteomic databases including Apis mellifera, geographically consistent plant, bee pathogens such as deformed wing virus, Varroa destructor, and Nosema ceranae, and plant pathogens. In order to ensure the specificity of the identified peptides, we applied a bioinformatics pipeline to compare peptide sequences to the entire RefSeq non-redundant database. We identified, for the first time, thousands of low-abundance peptides of diverse origin an example being Apis mellifera heat shock proteins, which are promising general stress biomarker, honeybee microbiome (Lactobacillus kunkeei, Gilleamella apicola) and pathogens (deformed wing virus, Varroa destructor, and Nosema ceranae), pollen (Profilin from Helianthus annuus), nectar, and plant pathogens (Sphingomonas sp).The present study showed that honey can be used as a tool for honeybee diagnostic disease and environmental biomonitoring. Through the use of functionalized affinity particles, we overcame the sensitivity problem that affected proteomics measurements in the honey and identified thousands of new honey peptides. In the future, we plan on expanding the number of samples to more diverse geographical areas and to follow hives longitudinally in order to correlate honey proteomics to disease status and response to treatment.

P1619/B756

Incorporation of Engineered Quorum Signaling to Control Biomaterials Based Upon Bacterial Biofilms.

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Prokaryotic biofilms rather than planktonic cells constitute the predominant form of prokaryotic life in nature. Despite the ubiquity of biofilms and their immense potential for bioengineering applications, ranging from bioremediation and nanomaterials manufacturing to their use as living materials, they have not been the focus of applications within the field of synthetic biology. To render biofilm utilization more feasible within synthetic biology, there is a need for a versatile and holistic toolkit to position and control biofilms precisely. To address this need, we have expanded upon the technique of biofilm

lithography pioneered by Jin and Reidel-Kruse (PNAS, 2018) through the incorporation of engineered quorum signaling within the biofilm. We will demonstrate additional functionality by tying biofilm maintenance, and propagation to the reception of these quorum signaling molecules. This approach can be used to limit the propagation of a biofilm, as the biofilm will only be maintained and propagated in regions where the local concentration of HSL reaches a certain threshold. With our toolbox, we have demonstrated that we are able to adhere to an inner layer of bacterial "sender" cells, which produce a quorum sensing signal that is able to be detected by an outer layer of bacterial "receiver" cells. Upon detection of the quorum signaling molecules, the receiver cells are able to express varied gene(s) of interest. As the sender and receiver circuits are constructed via 3G assembly, they are incredibly modular, and rapidly constructed. Currently we have demonstrated functionality of our toolbox through the assembly of a LuxI-based sender circuit, and a PLux/LuxR based receiver circuit with MScarlet-I as an output.

P1620/B757

Metabolic Stress Augments the Production of Advanced Glycation End Products Posttranslational Modifications on Proteins from Cellular Pathways Involved in Antigen Processing and Epitope Selection in Mouse Dendritic Cells.

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Increased oxidized ribose, glucose, glyoxal and methylglyoxal concentrations are observed in several metabolic diseases including diabetes and metabolic syndrome, mediating the non-enzymatic protein glycation and carbonylation followed by protein unfolding, cross-linking, and aggregation. In this presented research, we hypothesized that the protein posttranslational modifications induced by glycation and glycoxidation, i.e., the advanced glycation end products (AGE), will damage the immunological mediated pathways in mice models of T2DM metabolic syndrome. To test our hypothesis, we analyzed the AGE-modified proteomes and the immunopeptidomes eluted from MHC-II receptors using the CD11c+ purified primary dendritic cells from healthy controls C57BL/6, and *Ob/Ob* obese mice, models of T2DM. In addition, we validated the AGE-modified proteins in mouse JAWS dendritic cells treated with glucose concentrations (15-20 mM) found in the sera of diabetics. The peptides bearing AGE-amide adducts were sequenced by bottom-up label-free MS/MS that employed a QExactive HF quadrupole mass spectrometer. The immunopeptidomes were sequenced using the top-down MS/MS and an Orbitrap Fusion Tribid mass spectrometer using a combination of data-dependent (DDA) and data independent acquisition (DIA) methodologies. Bioinformatics and label free quantitative (LFQ) analyses highlighted the significant differences in the quantitative proteomic and peptidomic expression profiles among several cellular pathways affected by AGE, including endosomal maturation, antigen presentation, and processing machinery. An alysis of the type of AGE-PTM accumulated in the proteomes from Ob/Ob and JAWS dendritic cells treated with 20 mM glucose ranked the site-specific carboxymethylation of lysine (CML) as the most abundant AGE followed by formylation of lysine and carboxymethylation of arginine (CMA). Further mapping of PTM on the in *vitro* glycated human MHC II allowed the identification of key AGE-modified amino acids residues involved in peptide epitope binding. Overall, these results highlight the potential role for AGE-PTM accumulation in the pathophysiology of immune responses.

P1621/B758

The Reactome Pathway Knowledgebase: Curation and Visualization of Variants, Drugs, and Understudied Human Proteins.

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Pathway databases are a powerful bioinformatics tool for organizing and analyzing very large data sets, linking knowledge at a molecular level to more precise and effective diagnoses and treatments of human diseases. Reactome (https://reactome.org/) is an open-source, manually-curated and peer-reviewed knowledgebase of human reactions, pathways and biological processes. Our knowledgebase now comprises 12,608 human reactions organized into 2,287 pathways involving 11,040 proteins encoded by 10,860 different human genes, 2,078 small molecules (of which 222 are drugs), and 12,335 complexes. These annotations are supported by 30,398 literature references. We have curated a broad range of major disease processes that arise from somatic or germ-line mutations. To date, over 2000 unique variant annotations (for 305 proteins) have been associated with disease-variant forms of 968 reactions and other reactions that capture the effects of small molecule and drugs on these disease processes. Variants are chosen for annotation based on guidelines and data from ClinGen and COSMIC, and annotated with Disease Ontology terms. The annotation of small molecules, chemicals, protein or RNA drugs, are now cross-referenced to IUPHAR, PubChem or ChEBI allowing us to model the effects of therapeutic intervention on disease and normal pathways. As part of the Illuminating the Druggable (IDG) program, we have undertaken the role to project understudied proteins in the context of biological pathways to infer potential functions of these proteins. Reactome thus provides powerful, intuitive and reusable tools for visualization, interpretation and analysis of pathways to support basic and clinical research, genome analysis, modelling, systems biology and education.

P1622/B759

Screening of Tyrosine Motifs Which Can Induce Efficient Cell Proliferation and Its Development.

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Regulating induction of cell fate such as proliferation, differentiation and cell death is important technology for any cell-regulating technology such as regenerative medicine. Cell fate is induced and regulated by many mechanisms within a cell and one of the most significant one is signal transduction. In many cases cell fate has been controlled by adding sophisticated combinations of cytokines and chemicals. But these approaches depend on receptors which are endogenously expressed within a cell. To overcome this limitation, we have developed a technology of constructing artificial receptors which can regulate cell fate such as proliferation and differentiation. One of our approaches is to engineer a tyrosine motif, which is a part of the intracellular domain of receptors and acts as a binding region with intracellular endogenous signaling molecules. Here we show a new method for selecting artificial receptors which can induce cell proliferation in an efficient way by introducing random mutations to residues surrounding a tyrosine motif. Since this surrounding sequence is known to be important for their binding affinity against signaling molecules, in this way we can develop a *de novo* receptor library. In this study, we made such a receptor library using a signal transducer and activator of transcription 1 (STAT1) binding motif by randomizing 3 residues surrounding the central tyrosine residue. We

introduced this library into a cell line and after screening, several clones were obtained, all of which was shown to induce ligand-dependent cell proliferation. A signaling analysis revealed that different patterns of the activation of signaling molecules were successfully induced by this library. Interestingly, a key residue was found to be important for induction of efficient cell proliferation. As a conclusion, we succeeded in development of phenotypic screening of tyrosine motifs which can induce efficient cell proliferation.

P1623/B760

Recording Calcium in *Vivo*: Using a DNA-based Memory Device to Record Ca^{2+} Signaling in Mammalian Cells.

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Calcium (Ca^{2+}) ions are essential in nearly every aspect of cell biology, from early development to wound healing. As a ubiquitous messenger in cell signaling, control of intracellular calcium levels is tightly regulated within the cell, and complex spatial and temporal dynamics of calcium ion levels in the cell have far reaching effects on gene activation. This has driven efforts to track Ca^{2+} dynamics in cells; however, methods to do so in the past decades have relied on using Ca^{2+}-sensitive probes and live-fluorescent imaging, which may potentially disrupt Ca^{2+} dynamics and consequent gene expression. We have employed several approaches to tracking Ca^{2+} activity in *vivo*. First, we have used fluo-4 in primary neuronal cell cultures. We have also utilized GCaMP to measure intracellular Ca^{2+} levels in whole *Xenopus* embryos. Limitations to both of these approaches include photobleaching effects and fluorescence microscopy of live cells. In order to obviate the need for a fluorescent-based readout, we have aimed to construct a DNA-based memory device in order to record intracellular Ca^{2+} levels in mammalian cells by relying on sequencing output of a targeted gene locus. This transcriptionally activated recording can be multiplexed with other cellular events in order to investigate the relationship between intracellular calcium dynamics and its consequent effects on key gene regulatory pathways. Utilizing a CRISPR-mediated analog multi-event recording apparatus (CAMERA), an engineered Ca^{2+}-responsive promoter with nuclear factor of activated T-cells (NFAT) response elements is placed upstream of an engineered base editor, which will generate C•G to T•A mutations at a designated locus on the CCR5 gene in the HEK293T genome. Following induction of intracellular calcium levels, DNA-based recording of dynamic Ca^{2+} levels is detected with high-throughput sequencing of the target genomic locus. Results with traditional calcium indicators display clear differences in the calcium activity of neuronal precursors (high frequency, low amplitude activity) compared to neuronal cells committed to differentiation (low frequency, high amplitude); these results are being compared with those obtained from the memory-based recording system.

P1624/B761

Exploring the Hedgehog Signaling Pathway Using Unnatural Amino Acid Mutagenesis.

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Hedgehog (Hh) is a secreted signaling protein involved in tissue patterning during embryogenesis and stem cell regulation in adults. Misregulation of the Hedgehog pathway leads to developmental defects and can be a driver for cancer. The extracellular Hh ligand can directly bind to a number of cell surface

receptors, including its canonical receptor Patched (Ptc), and co-receptors Boc, Cdon, Hhip, and Gas1. However, how Hh interacts with these proteins is not fully understood. Unnatural amino acid mutagenesis is a powerful approach through which non-natural amino acids are introduced within proteins in a site specific manner through the use of a repurposed sense codon (UAG) in the mRNA of interest and an orthogonal $tRNA_{UAG}$ and a tRNA aminoacyl tRNA synthetase that recognises $tRNA_{UAG}$ in the mRNA. The use of cross-linking amino acids, such as Benzoyl phenylalanine, into proteins allows the identification of direct binding partners and allows mapping of the interaction site distances with sub-nanometer precision. We have applied this powerful methodology to first validate the known interaction sites of the Hh ligand with its receptors Ptc and Hhip, respectively, and find that the cross-linking studies are largely consistent with the published X-ray and cryo-EM data on Hh-Ptc and Hh-Hhip interactions, while also identifying new interaction sites. We are now using this approach to precisely understand the different domains on Hh that interact with other Hh receptors such as Boc, Cdon, Gpc3, and Gas1.

P1625/B762

Engineered Adhesin Library for Construction of Bacterial Biofilm-based Biomaterials.

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The majority of prokaryotic cells live in multicellular consortia known as biofilms. Biofilms are typically associated with increased pathogenicity and environmental effects such as biofouling. However, due to their ubiquity and outstanding bioengineering potential, bacterial biofilms may be repurposed as living biomaterials to perform useful functions ranging from bioremediation to clinically-relevant sensors. Design and production of such synthetic biology applications is hindered by a lack of well-characterized parts for biofilm engineering and control. To ensure robustness and control of biofilm-based biomaterials, we have constructed a library of biofilm-forming and strengthening adhesins for use in E. coli biofilms. This library builds upon pDawn-AG43, a plasmid designed by Stanford bioengineers (Jin and Riedel-Kruse, PNAS, 2018) for blue-light induced biofilm formation. The adhesin utilized in pDawn-AG43, E. coli outer membrane protein antigen 43, forms spatially-controlled but relatively weak biofilms when induced. Using both genomic PCR and DNA synthesis, we obtained three amyloid fibers to complement and even replace AG43. These include: curli fibers (the main proteinaceous component of native E. coli biofilms), SaSuhB (a macroscopic fiber produced by S. Aureus), and Functional Amyloid in Pseudomonas, or FAP (an amyloid fiber that contributes to biofilm formation in P. Aeruginosa). Such fibers were placed in 3G Assembly-compatible format for the construction of chemical and light-induced circuits, and fiber expression was quantified via Congo Red staining. For both SaSuhB and FAP, fiber expression was further visualized via microscopy. An alysis of all three adhesins has shown robust, controllable adhesion and biofilm formation, which expands upon the available tools for biofilm formation and control.

P1626/B763

Stem Cell Manufacturing Using Sustained-release Nano-micro Carrier in Single-use Bioreactor Systems.

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In the last decade, micro-carriers and bioreactor system has been developed for clinical trials in many companies because stem cell-based therapy demand a large number of cells. However, micro-carriers

were very high cost due to surface modification for cell attachment. Here we have developed a nano-microcarrier and single-use bioreactor system for effective expansion of adult stem cells. The nano-microcarrier includes open porous silica nanoparticles (OSNs)-conjugated polystyrene microbeads (PS-OSN), which is able to load the growth factors or cell attachment factors efficiently and to control the sustained release. As a result, we have established an optimal condition for adult stem cell cultivation using PS-OSN carriers in single-use bioreactor system. Also, a stable and effective expansion yield of the stem cells was achieved compared to commercialized micro-carriers. In addition, we confirmed the pluripotency and proliferative capacity of stem cells compared to conventional monolayer cultivation. Our results will apply to the cost-effective tool in downstream for stem cell-based therapy.

Engineering Tissues and Organs

P1627/B764

Recapitulating Biological Signaling Scenarios with Spatiotemporal Control Using a Multiplexed, DNA-Patterning Approach.

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Dissecting the complex action of the biological signals that orchestrate single-cell fate decisions as well as tissue function/dysfunction is challenging due to the spatiotemporal parameters modulating their actions. This complexity is further compounded by the fact that these signals—soluble ligands, extracellular matrix-sequestered cues, and cell surface-presented cues—comprise a larger network in which multiple signals are coordinating with each other across time and space. In order to model and investigate such dynamic signaling scenarios in *vitro*, we have engineered a DNA-based patterning platform that imparts unprecedented spatial and temporal control over individual and multivariable signaling scenarios. Specifically, we utilize photolithography to generate a patterned photoresist (PR) template that guides the conjugation of DNA oligonucleotides onto a surface. The PR-template can be stripped and a new one patterned without damaging the initial DNA layer. As we demonstrate, we can subsequently register and layer up to 10 orthogonal DNA strands, remarkably without sacrificing lithographic resolution. The resulting, multiplexed DNA patterns are highly functional and can hybridize to complementary DNA sequences that have been tagged to cells and/or solid-phase signaling ligands. The precision of the DNA patterns is thus transferred to the assembled in *vitro* model, imparting micron-scale control in recapitulating single-cell to bulk-tissue signaling environments. To demonstrate our platform's utility in de-coupling the spatial dependencies of biological signals, we investigated how two competing niche signals, fibroblast growth factor-2 (FGF-2) and ephrin-B2, coordinate spatially to instruct adult neural stem cell fate decisions at the single-cell level. By modulating their spatial presentation, we observe a strong spatial bias toward FGF-2 that, in some unexpected cases, did not translate to the predicted phenotype of high proliferation and maintenance of stem cell identity but rather high neuronal differentiation. To achieve temporal control within our platform, we demonstrate various nuclease-based strategies to modulate the action of DNA-hybridized signals. With these spatiotemporal capabilities in-hand, our DNA-directed approach provides opportunities to investigate the underlying signaling mechanisms of key processes across a wide range of biological disciplines.

P1628/B765

Electrophysiological Property Evaluation of Reconstructed Cardiac Tissue-like Cell Community.

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Cardiac tissue in vivo has a structure that cardiomyocytes (CMs) and fibroblasts (Fibs) are regularly oriented. While, cardiac cell sheet obtained by dispersed culture has multi-directional sarcomere structure and random contacts of CMs and Fibs. We previously reported that dispersed culture of cell obtained from cardiac tissue had made conduction velocity slower. Therefore, there is an issue that they show the different functions such as contractile force and electrophysiological property. It is said that cardiac cell sheet transplantation is adopted as a therapeutic discipline; however the minute and detailed effect is still incompletely understood. In this study, we obtained cardiac tissue-like structure on a substrate with agarose microchamber, where Fib network is localized between two Linear-CM communities and mediates their signal conduction. The CM-Fib-CM network resembling cardiac tissue in vivo was reconstructed using micro patterning of agarose gel and conducted membrane potential measurement using multi electrode array system. The condition of cell community was divided into 3 stages based on Fib proliferation: network consisting of CM only, network which CMs and Fibs localized in designated spots, and cardiac tissue-like cell community. Then we demonstrated the function of cell community and compared between 3 stages. Firstly, we found that there was no significant difference between 3 stages in Inter-spike interval (ISI) which is the time between subsequent action potentials of Na^+ Infflux. It means these 3 types of cell community had almost the same spontaneous rate and pacing speed. Secondly, we focused on Coefficient Variation (CV) in order to evaluate stability of cell community. As a result, cardiac tissue-like cell community showed the lowest CV value and the most stable beating. It is thought that almost the same ISI between groups resulted from cell orientation/structure and proportion of CM and Fib. Cell communities demonstrated in this study are reconstructed using AMC, therefore cell orientation was unidirectional and CM networks and Fib ones are located in designated spots. Moreover we assumed that Fibs had an effect on cardiac stable beating. Further studies are required in order to reveal the role of Fibs in cardiac tissue and we have been analyzing also in terms of conduction velocity of each cell community.

P1629/B766

Scaffold-based Biosensors - New Tools for Studies of Three-Dimensional Tissue Culture Models.

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Cells heavily depend on presence of the extracellular matrix, which is frequently used in re-creating in *vivo* conditions in 2D/ 3D tissue culture, tissue engineering and transplantation applications. In turn, analysis of extra- and pericellular metabolites, dissolved oxygen and other biomolecules can inform on cell physiology, viability, mitochondrial function and bioenergetics, without the need of the intracellular measurements. A broad selection of various scaffolds and matrices employed in tissue engineering prompts design of multi-functional 'hybrid' matrices, which not only support the cell function but also provide additional functionality. Here, we present an approach enabling design of biosensor matrix materials, based on use of luminescent dyes, nanosensors and bioengineered fluorescent proteins. First, organic polymer-based scaffolds such as polystyrene or PCL can be modified via impregnation of Pt(II)-porphyrin hydrophobic dye and become O_2-sensitive. Alternatively, various collagen-based matrices display aggregation and retention of water-soluble O_2-sensitive nanosensors; this feature can be exploited for producing biosensing xenotransplants or modifying Matrigel for organoid and related

structures. Third approach is based on the use of inventory of protein-binding domains and pH, $Ca^{2+}$and other biosensor proteins. Here, we present new data on production of cellulose and collagen-based biosensor matrices compatible with measurement of extracellular pH in 3D cultures of colon cancer cells and mouse intestinal organoids. Such biosensor matrices are compatible with conventional fluorescence microscopy and enable quantitative measurements based on the intensity or fluorescence lifetime (FLIM, 1.5-2.2 ns) or phosphorescence lifetime (PLIM, 10-50 ʋs range) imaging readouts. In a proof-of-concept study, we produced pH-sensing cellulose scaffold based on cyan fluorescent protein and used it for monitoring pH changes associated with action of mitochondrial uncoupler on the mouse intestinal organoids. We found that recorded changes in pH were concomitant with the effect on cell oxygenation due to action on the mitochondria. We also demonstrate how the Matrigel matrix can be modified with O_2-sensitive nanoparticles in order to monitor oxygenation in vicinity of live intestinal organoids. Collectively, presented approach provides means for label-free quantitative analysis of metabolism in the tissue-engineered constructs.This work was supported by the Science Foundation Ireland (SFI) grants 13/SIRG/2144 and 18/IF/6238.

P1630/B767

A Validated Test for Assessing Cell Viability in Scaffolds: Measuring Atp/dna for Cells in Hydrogel Scaffolds.

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Abstract: Tissue engineered medical products are often comprised of cells suspended in a 3D scaffold. The mechanism of action may require that the cells are viable to regenerate tissue or to secrete bioactive factors that induce tissue repair. There is a need to be able to reliably measure cell viability in scaffolds as a quality attribute of the product. Traditional biochemical cell viability assays assess membrane leakiness, metabolic activity or the presence of cellular components that are indicative of cell viability (such as ATP). The scaffold may interfere with these assays in unknown ways, such as by impeding the diffusion of assay components or by altering reaction kinetics, making it difficult to interpret results. We have developed a model scaffold-cell-assay system for assessing cellular viability. For this assay, moles of ATP per gram of DNA was measured using a luminescence ATP assay and a fluorescence DNA assay. A glucose-based, polysaccharide, shear-thinning hydrogel was used for the scaffold which was seeded with human Jurkat cells. Control experiments were conducted with gels spiked with ATP and DNA to make sure that, 1) at the hydrogels do not interfere with the assay, 2) the gel components do not contain ATPases and 3) the assay reactants could adequately diffuse into and out of the hydrogels to obtain reliable results. In order to validate results, in situ results for gels were compared to results from gels that were disassembled by shear-thinning pipetting action to release the contents. Results from these studies are guiding an interlaboratory study to support an ASTM Standard Test Method for Measuring Cell Viability in Scaffolds. The model scaffold-cell-assay system may also be used as a test bed to assess advanced measurements of cell viability in scaffolds, especially less invasive or 3D imaging techniques such as electron paramagnetic resonance imaging of oxygen consumption or photoacoustic imaging of live cell distribution within 3D scaffolds.

P1631/B768

Synchronization of Beating between Dispersed Cardiomyocytes and Cardiac Tissue Piece.

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Myocardial infarction is a famous disease in the heart. Transplanting cardiomyocytes sheet onto infarcted area is expected as a new treatment for heart disease. A new method using cardiomyocytes sheet has been developed for the treatment of the ischemic heart disease. However, the mechanism of beating synchronization at transplantation between cardiomyocytes sheet and heart is still unclear. As post-transplant electrophysiological signals couldn't be measured continuously in *vivo* heart, in *vitro* model of transplantation between cardiomyocytes sheet and heart were reproduced on Multi Electrode Array (MEA) by using dispersed cardiomyocytes and cardiac tissue piece. Recently, MEA system measure of extracellular potential and expected to evaluate the beating synchronization process. To reveal the synchronization process between autonomous beating dispersed-cardiomyocytes and cardiac tissue piece with the oriented impulse conducting system, we measured and analyzed the extracellular potential of these cardiomyocytes for beating interval. For analysis, the sodium peaks' time at each sample was of tainted. Then, Inter spike interval (ISI), the beating interval, can be captured. Also, the beating time difference between dispersed cardiomyocytes and cardiac tissue piece were calculated. As a result, we obtained two kinds of data. Dispersed cardiomyocytes having long and stable ISI became pacemaker-region, and on the other hand, a dispersed cardiomyocytes or cardiac tissue piece having short and stable ISI became pacemaker-region. Therefore, we clarified that the stable beating rate was dominant for was pacemaking regardless of beating speed and whether dispersed cardiomyocytes or cardiac tissue piece. In conclusion, stable beating function was a more important factor than oriented structure in beating synchronization. When the cardiomyocytes sheet is to be used as a treatment method, cardiomyocytes sheet will be pacemaking after transplantation if the beats of cardiomyocytes sheet is more stable than it of heart. Moreover, it was suggested that to transplant cardiomyocytes sheet having stable beating might be induced the arrhythmia of heart.

P1632/B769

Thick, Functional, 3D Liver Tissue Microenvironment Model.

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It is estimated that 90% of pharmaceuticals fail during clinical trials, despite success in animal studies. In order to alleviate this bottleneck two main strategies are needed. 1. Tissue throughput, repeatability, and automation needs to be addressed to enable clinical level production. 2. To reduce these failures, there is a strong need for in vitro models that can recapitulate the 3D in vivo environment using humantissues.In this study we have developed a robust 3D liver model leveraging 3D printing and sacrificial moulding techniques to create a thick (≤ 1mm), high-cell density hepatic tissue. To accomplish this, vertical pillars of a sacrificial hydrogel were printed within individual wells of a 96-well plate to ultimately form channels for media exchange. 10-20 million primary human or rat hepatocytes/ml, mixed with varying numbers of non-parenchymal cells (NPCs), with or without isolated, adipose, microvessels were suspended in proportionally small amounts of type 1 collagen. The hepatocyte/NPC/microvessel collagen slurry was dispensed around the sacrificial pillars, which were then washed out after the tissue sets.Culture supernatants were analysed for the production of urea and the absence of lactate dehydrogenase (LDH). Urea secretion levels were 2.8 times higher at day 7 than day 2, demonstrating that hepatocytes maintained their functionality during longer term culture in

a thick 3D tissue format. LDH values were over 100 times lower at day 7 than day 2. This indicates that despite some expected cell death following hepatocyte thawing and seeding, the construct quickly stabilized and maintained viability through day 7. Histological analysis confirmed a high cell density at day 7 and negligible necrosis. Imaging of the hepatic tissue constructs with the GE INCell 6500 high content cell imaging system showed preservation of the original tissue and wall structure of the exchange channels as well as cell heterogeneity within the thick tissue construct.In addition to developing a fabricated tissue construct that creates an hepatic-like tissue environment in a throughput format, we are using this liver construct to develop automated tissue fabrication, integrating our BioAssembly® platform with the GE in Cell scanner to enable commercial-level production of sophisticated 3D tissue models with the potential to decrease drug discovery timelines and improve bench to bedside care.

P1633/B770

Modulation of Cartilaginous Matrix Characteristics by Mechanical Dose - Optimization of in Vitro Fibrocartilage Reconstruction.

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For reconstruction of fibrocartilage tissue, induction of tissue alignment and cartilaginous matrix production is essentially needed. Although tensile stimulation is the most effective approach to induce tissue alignment, recent studies showed that mechano-transduction interferes with the signaling pathway which is upstream to chondrogenesis. In our study, sheet-cultured meniscal chondrocytes (MCs) were rolled-up to form cable-shape tissue construct. When the roll-up cultured MCs were subjected to static tension (10%), they resulted in severely attenuated glycosaminoglycans (GAGs) production even in the presence of TGF-β3, a chondrogenic growth factor. To minimize the inhibitory effect of mechano-transduction on cartilaginous matrix production, the dose of tensile stimulation was modulated by tension-release after the induction of tissue alignment by static tension. The mechanical dose of 10% tensile stimulus for more than 7 days subjected to MCs resulted in maintenance of highly aligned tissue upto 5 days after tension release. When the roll-up cultured MCs were subjected to dose-modulated tensile stimulus during 3 weeks of chondrogenesis, matrix production was potentiated in contrast to the cells kept in static tension state for 3 weeks. Examination of nuclei angle showed that application of 7 days of static tension resulted in maintaining the tissue alignment in majority of the cells even after the tension release. The proportion of cells showing nuclear translocation of YAP increased in mechanical dose-dependent manner. The mechanical dose-modulated tissues subjected to chondrogenesis not only maintained tissue alignment but also produced cartilaginous matrix composed of type I collagen and GAGs matrix, which was evidenced by immunohistochemical staining and histological analysis. In conclusion, these finding suggest that the optimization of tensile stimulus can modulate the characteristics of cartilaginous matrix production, which can lead to the successful reconstruction of fibrocartilage.

P1634/B771

Application of Recombinant Collagenase to Tissue Dissociation and Cell Recovery.

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Purpose: Collecting pure and qualified cells from human donor is a critical but challenging process that hinders successful tissue-engineering and cell biology researches. Mostly, collagenase, derived from *Clostridium histolyticum,* is utilized to isolate cells from tissue. However, crude collagenase contains various neutral proteases which can cause cellular damage during tissue dissociation. Therefore, we produced recombinant collagenase from *Escherichia coli* through bioprocessing technology. **Materials and methods:** Collagenase potency assay of recombinant collagenase was performed using FALGPA peptide as a substrate (synthetic short polypeptide specifically recognized by collagenase). Based on potency assay, proper unit of recombinant collagenase was treated to lung, kidney and spleen tissue from mice to recover immune cells and bovine cartilage to obtain chondrocytes. Three commercialized enzymes were used as a control representing crude collagenase. To compare cell quality, genetic and CD markers were measured by real time PCR and flow cytometry. **Results:** Comparing to control collagenases, immune cells recovered by recombinant collagenase showed fewer numbers, while population of T cell, B cell, NK cell, NKT cell, myeloid and mononuclear phagocyte was similar in both collagenases. However, population of immune cells from spleen obtained by recombinant collagenase showed more even distribution compare to crude collagenase derived ones. Chondrocytes, on the other hand, showed similar recover rate with both cases but ones gained by recombinant collagenase exhibited healthy chondrocyte-like characteristics in regard of genes (*Aggrecan*, *Col1a1*) and surface CD markers (CD14, CD44). **Conclusions:** Although tissue dissociation capacity of crude collagenase in lung, kidney and spleen was confirmed to be superior to the recombinant one, distribution of recovered immune cells was mostly similar in both cases. In particular, unlike other two tissues, spleen-originated immune cells with recombinant collagenase showed more even distribution. Since splenic immune cells are used as a reference for immune cells extracted from organs, it is advantageous to present a clear standard using recombinant collagenase. In addition, chondrocytes recovered by using our recombinant collagenase were determined to maintain their original characteristics better than crude collagenase-isolated ones.

Development and Morphogenesis: Organogenesis and Body Size

P1635/B773

Connectivity of the Glomerular Capillary Network.

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The capillary network of the kidney glomerulus performs the initial filtration of blood in the nephron. It begins as a single capillary loop, but it is not known how the network forms or how short cuts between afferent (input) and efferent (output) capillaries are prevented. The ATUM tape collector method was used to image 500 nm thick serial sections of mouse glomeruli at 50 nm per pixel. 12 glomeruli were reconstructed. Afferent capillaries were thicker in cross section than efferent capillaries indicating that capillary diameter adapts to flow volume. A no cross zone bisecting the glomerulus between the afferent and efferent capillaries demonstrates the lack of short cuts. The connectivity was determined by a virtual reality system, and was analyzed by network theory, a well established mathematical tool. The glomerulus is not a "planar graph" which shows that it does not form from longitudinal splitting of capillaries. Even though the 12 glomeruli vary five fold in branch number, the number of branches in the shortest path is approximately the same for all of the glomeruli. This may indicate that later branches are added on the side of the glomerulus opposite from the input and output capillaries.

P1636/B774

Loss of Hhex Causes Absence of Hepatopancreatic Ducts but Formation of an Intrahepatic Intestinal Tube Due to Expansion of the *Cdx1b* and *Pdx1* Expression.

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The hepatopancreatic duct (HPD) system links the liver and pancreas to the intestinal tube and is composed of the extrahepatic biliary duct, gallbladder and pancreatic duct. Haematopoietically-expressed-homeobox (Hhex) protein plays an essential role in the establishment of HPD, however, the molecular mechanism remains elusive. Here we show that zebrafish *hhex*-null mutants fail to develop the HPD system characterized by lacking the biliary marker an nexin A4 and the HPD marker *sox9b*. The hepatobiliary duct part of the mutant HPD system is replaced by an intrahepatic intestinal tube characterized by expressing the intestinal marker fatty-acid-binding-protein 2a (*fabp2a*). Cell lineage analysis showed that this intrahepatic intestinal tube is not originated from hepatocytes or cholangiocytes. Further analysis revealed that *cdx1b* and *pdx1* were expressed ectopically in the intrahepatic intestinal tube and knockdown of *cdx1b* and *pdx1* restored the expression of *sox9b* in the mutant. Chromatin-immunoprecipitation analysis shows that Hhex binds to the promoters of *pdx1* and *cdx1b* genes to repress their expression. We therefore propose that Hhex, Cdx1b, Pdx1 and Sox9b form a genetic network governing the patterning and morphogenesis of the HPD and digestive tract systems in zebrafish.

P1637/B775

Lysosome Rich Enterocytes (LREs) Mediate Protein Absorption in the Vertebrate Gut.

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The guts of neonatal mammals and stomachless fish have a limited capacity for luminal protein digestion, which allows oral acquisition of antibodies and antigens. However, how dietary protein is absorbed during critical developmental stages when the gut is still immature is unknown. Here, we show that specialized intestinal cells, which we call lysosome-rich enterocytes (LREs), internalize dietary protein via receptor-mediated and fluid-phase endocytosis for intracellular digestion and trans-cellular transport. We identify a conserved endocytic machinery in LREs, composed of the scavenger receptor complex Cubilin/Amnionless and Dab2, that is required for protein uptake by LREs and for growth and survival of larval zebrafish. Moreover, impairing LRE function in suckling mice, via conditional deletion of Dab2, leads to stunted growth and severe protein malnutrition reminiscent of kwashiorkor, a devastating human malnutrition syndrome. These findings identify digestive functions and conserved molecular mechanisms in LREs that are crucial for vertebrate growth and survival.

P1638/B776

Nitric Oxide Signaling in Regulation of Normal Heart Loop Morphology.

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Asymmetric elevation Ca2+ signaling at the left side of the Hensen’s node in early embryo development is essential for normal right side heart looping and consequently normal heart development. As in all vertebrates, the first organ to develop is the heart and an early indicator of a properly forming heart is a

dextral C-shaped heart loop. In the chicken embryo cardiogenesis begins in bilateral heart fields within the splanchnic mesoderm closely associated with the endoderm. Both are created by the migration of cardiac progenitors from the anterior third region of the primitive streak at gastrulation (HH3-4 stage embryos).The progenitors migrate lateral and anterior in the cranial mesoderm to be in parallel with the embryo midline and slightly anterior to Hensen's node.There an oblong region of cardiac progenitors is formed creating the bilateral heart fields which consist of the first heart fields(FHF) and secondary heart fields (SHF). Endocardial tubes then form showing cellular cardiac differentiation and progression of the nascent heart that forms in an anterior-posterior axis in the embryo as a heart tube at HH10 stage embryo and loops at HH13-15. When calcium levels are elevated on the right side or blocked on the left side of Hensen's Node, both result in 30% of embryos with situs inversus (reversal of the heart loop). Nitric oxide (NO) formation also depends on elevation of calcium and can promote cardiomyocyte proliferation. However the role of Nitric Oxide (NO) in regulation of heart looping is unknown. Our objective for this study was to investigate NO signaling effects on early heart tube looping as a regulated process of morphogenesis in the chicken embryo. We hypothesized NO involvement in cardiac looping may be by its ability to regulate cardiomyocyte proliferation and under absence of NO may cause a looping defect due to absence of sufficient cardiogenic cells. To investigate, an acrylic bead soaked in L-NAME, a competitive inhibitor of nitric oxide synthase(NOS), or DETA-NONATE, an NO donor, was placed to the right side of Hensen's Node to block or elevate NO signaling. Our findings show 80% abnormal heart tube formation with situs ambiguous, a range of morphological heart looping defects, when NO signaling is disrupted. These results suggests that the calcium elevation on the right side functions in the activation of NOS for NO production and signaling, possibly by its canonical pathway, to regulate in the normal heart loop. NO signaling in early embryo development is not well understood and it's role in cardiomyocyte proliferation may also need to be studied to fully understand cardiogenesis. NSF DBI-1548297

P1639/B777

Single Cell Dynamics Driving Vertebrate Heart Valve Interstitial Layer Morphogenesis.

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Heart valves are critical structures that close the cardiac lumen to prevent retrograde blood flow. Within the valve, specialized cells called valve interstitial cells (VICs) secrete extracellular matrix (ECM) that confers biomechanical strength to withstand blood flow. Considering that many hereditary congenital heart valve defects in humans arise due to aberrant VIC formation, it is crucial to better understand the mechanisms driving this process. As VICs arise later in development, the in *vivo* dynamics that drive their morphogenesis have not been studied in live animals or at single cell resolution. Using the zebrafish atrioventricular (AV) heart valve, we provide the first detailed analysis of the cellular processes that lead to the establishment of the VIC layer. Initially, a group of endocardial cells (ECs) at the AV canal collectively migrate to establish pre-valvular leaflets at the embryonic stage. Some of these cells then undergo endothelial-to-mesenchymal transition (EMT) and invade the space between two EC monolayers to become the first VICs. Lineage tracing shows that VIC establishment also comprises a smaller contribution from neural crest cells, which persist throughout development. Mature VIC establishment further includes a peak of cell proliferation at the juvenile stage, and a progression of ECM components from a pro-migratory to an elastic profile. Furthermore, the molecular regulation of VIC establishment has been poorly understood. We establish and analyze the first zebrafish model with

defective VIC development: mutations in the gene encoding the NFκB-related transcription factor Nfatc1 lead to significantly fewer VICs, causing valve malformations and retrograde blood flow. *nfatc1* mutants exhibit reduced VIC invasion and proliferation, as well as defects in ECM deposition. Interestingly, similar to symptoms in patients with heart valve defects, *nfatc1* mutants can survive to adulthood, but display severe morphological and functional cardiac defects. We found that Nfatc1 functions at least in part by regulating the expression of one of the EMT-promoting factors, Twist1, suggesting a complex transcriptional network that drives VIC invasion. In summary, our study uncovers the single cell dynamics and molecular signatures underlying VIC morphogenesis. With this unique animal model, we propose to determine the gene regulatory networks that mediate the invasive and proliferative behaviours of VICs.

P1640/B778

Evaluation of Regeneration After Cauterization as a Model for Cardiac Injury in Adult Zebrafish.

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Using the zebrafish with total regenerative capacity, we seek to standardize and describe the process of cardiac regeneration after cauterization. This kind of injury may replicate better the ischemic event that happens in humans. Thus, we describe the process of cardiac regeneration, as well as the electrocardiographic characteristics of zebrafish (*Danio rerio*) in a period of 90 days after cauterization injury (pc). The process is recorded after performing the cauterization injury on day 7, 15, 30 and 60 pc with the corresponding sham controls. At the described times, 5 animals per group are randomly selected for euthanasia and the hearts are preserved. These are evaluated through Masson-Goldner trichrome staining to describe tissue architecture and histopathological features. To evaluate the number of proliferating cardiomyocytes, phosphohistone 3, AuroraB, Phalloidin and cardiac troponine T inmunofluorescence is performed. In order to evaluate function, the electrical activity of the heart is determined for the injured as well as for the control groups on the same times postinjury. At the end point (90 days pc), these animals are also processed for histopathological and immunofluorescense analysis. Preliminary results suggest the loss of ventricular tissue architecture with necrotic regions, fibrotic deposits, and intense inflammatory response according to trichromic staining during the first 15 days after injury. At later times, 30 dpi, the necrotic and fibrotic tissue appear to be replaced, starting the restauration of the original structure. Previous works have shown delayed in the QT segment after injury. However, it has not been determined after cauterization. We expect that electrical function returns to baseline by 90 days pc. Similarly, we expect immunofluorescence shows re-entry into the cell cycle and mitosis of numerous cardiomyocytes not only in the margin of the lesion but in areas distant from it. Our work characterizes the cauterization injury, which has not been fully described previously for zebrafish. We consider, this model can give us a better approach to conditions of a cardiac ischemic event. Thus, bringing new elements to the knowledge of cardiac regeneration and translational research in search of new therapeutic approaches.

P1641/B779

The Titin Lifecycle: Sarcomere Dynamics and the Spatial Organization of Protein Turnover.

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The giant striated muscle protein titin determines sarcomere structure and elasticity. It integrates into Z-disc and M-band of the sarcomere and thus forms a continuous filament along the myofiber. Transport and turnover of titin provide a unique challenge to striated muscle cells, based on its size and multiple integration sites. We took a visual approach and followed fluorescently labelled titin in developing and adult myocytes to understand how titin is synthesized, transported, integrated into the sarcomere, released, and degraded. Our titin knockin mice express eGFP fused to titin's M-band region or dsRed attached to the Z-disc region and provide a unique view on titin mobility and turnover in cardiomyocytes and myotubes. We localized protein synthesis to the Z-disc by ribosome staining and titin RNA detection by smFISH and found that a significant amount of titin is not integrated into the sarcomere even in mature cells. Using fluorescent recovery after photobleaching, we documented an active exchange between the soluble pool and the sarcomere lattice. The underlying kinetics differ not only between Z-disc and M-band, but also between individual cells - suggesting intercellular heterogeneity of titin protein expression. Titin is distributed not only along the sarcomere, but also along the complete syncytium after the fusion of skeletal muscle myocytes. Here, we find an unexpected arrangement of titin at the Z-disc in developing skeletal muscle, but not in mature fibers of cardiomyocytes. Understanding the role of titin in sarcomere assembly and -dynamics can help understand heart and skeletal muscle remodeling in development and disease.

P1642/B780

Modeling Human Skeletal Muscle Development and Stem Cell Niche Formation in *Vivo*.

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We are interested in using human pluripotent stem cells to generate skeletal muscle progenitor cells (hPSC-SMPCs) for development of cell therapies. My previous work showed that increased myogenic ability resides in the ERBB3+NGFR+ fraction of hPSC-SMPCs. We developed a single cell RNA-Sequencing atlas of human PAX7+ cells across fetal, juvenile and adult which identifies that hPSC-SMPCs align between human fetal weeks 8-12 using diffusion map analysis. PAX7+ muscle cells fulfill different functional needs during myogenic development. Understanding how PAX7+ cells behave across different developmental states is critical to understanding their regenerative potential and to evaluating hPSC-SMPCs. We show that human fetal SMPCs and adult satellite cells (SCs) differ in their ability to make myofibers in *vitro* and in *vivo*; and fetal SMPCs have reduced ability to home to the SC niches of mdx-NSG mice compared to adult SCs upon engraftment. We have shown hPSC-SMPCs engraft to restore new myofibers similar to levels seen in uncultured fetal SMPCs. We also found hPSC-SMPCs fuse to form

hundreds small human-specific only myofibers in *vivo*. PAX7+ hPSC-SMPCs were primarily associated with these regenerating human-only myofibers. We found that human-only myofibers continue to grow over 60 days in *vivo*, and PAX7+ hPSC-SMPC associate under the basal lamina of these emerging myofibers over time. This work demonstrates for the first time that fetal and hPSC-SMPCs can be used as a model to study human myofiber formation and niche occupancy in *vivo*. We further hypothesized that human SMPCs did not efficiently home to mouse SC niches due to competition with mouse Pax7+ SCs. Using mdx-NSG Pax7$^{cre/ERT2}$ RosaDTA mice, we evaluated whether population-specific ablation of mouse Pax7 SCs would improve retention of engrafted human PAX7 cells. We found an increase in human PAX7 cells in mdx-NSG PAX7-DTA mice in human niches but not mouse SC niches. Our work suggests that donor cell niche homing occurs in newly regenerating fibers, and not to empty SC niches of established myofibers. Evaluating human niche formation over time will improve our understanding of how human muscle SC niches develop. This could improve our ability to generate *de novo* human niches and better support human PAX7 cells in *vivo* for cell therapy.

P1643/B781

Stim2 Splicing Variant Regulates Chondrogenesis.

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Ca^{2+} is a ubiquitous second messenger which plays essential roles in many cellular processes, including cell differentiation. Store-operated Ca^{2+} entry (SOCE), a major calcium regulation mechanism in non-excitable cells, tightly regulates dynamic Ca^{2+} signals. Stromal interaction molecule (STIM) senses ER Ca^{2+} depletion and induces Ca^{2+} influx by activating Orai Ca^{2+} channel located in the plasma membrane. Recently, we reported that STIM2 splicing variant plays a role in myogenesis. However, whether this STIM2 splicing variant is involved in chondrogenic cell differentiation remains elusive. Here, we show that STIM2 splicing variant is essential for chondrogenesis. Reverse transcription-polymerase chain reaction revealed increased expression of SOCE components in differentiating ATDC5 chondrogenic cells. To confirm the role of STIM2 splicing variant in chondrogenesis, we generated STIM2 splicing variant knock-out ATDC5 cell by CRISPR-Cas9 system. The deletion of STIM2 splicing variant showed the altered calcium level and delayed physiological processes such as proliferation, migration, and differentiation. These results provide the evidence that STIM2 splicing variant might play an essential role in chondrogenic ATDC5 cell differentiation. This study improves the understanding of cellular differentiation research, particularly mediated by SOCE.

P1644/B782

The Effects of Changing Serum Conditions on Myotube Formation in C2c12 Cells.

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C2C12 cells are an immortalized mouse myogenic stem cell line, a model for muscle development. Expression of MyoD, Cdk1, and Titin are good genetic markers for cell cycle, myogenesis and sarcomerogenesis, respectively. This study looks tat the effects of different growth media on myogenesis and gene expression during myogenesis. The various serum-based media used are Dulbeccos's Modified media base media supplemented with, either Fetal Bovine Serum (10% or 1%) or Horse serum Media (10% or 1%). In addition to these undefined media (serum) we also looked at defined media, PC-1 defined media (transferrin, insulin like growth factor. The effects of the different medias on C2C12 cell morphology and the gene expression of cdk1, MyoD and the titin isoforms was

determined. Cells were grown over a 12-day time course study with imaging, media change, and pelleting cells for mRNA isolation. Quantitative rtPCR was carried out to measure expression of specific myogenic genes and correlated to changes in morphology. In addition to the time course study a switch-back experiment was done on days 2 and 8 where the cells were changed from their experimental media back into the control 10% FBS. Images and pelleting of cell was done 2 days post-switch-back. The results shows changing culture conditions leads to alteration in cell morphology and genetic expression in C2C12 cells. Cells will proliferate and grow into normal myoblasts however; differentiation does not normally proceed. Myotubes are observed to differentiate but do not do so in the normal fashion or density. In normal differentiation myotubes are observed in long dense sheets of muscle fibers. During myogenic differentiation there is observed an increase in mitochondrial DNA relative to nuclear DNA. In the switch-back experiments cells in the cultures did not differentiate normally and were at low densities. When switched back into 10% FBS the density increased and myotube development begins to resemble the controls. To confirm that the cells at confluency are no longer in the cell division cycle and have started differentiation, flow cytometry analysis on pre-confluent, confluent, and post-confluent C2C12 cells was done. These results agree with the previous observation of a dramatic decrease in cdk1.

P1645/B783

Human Pulmonary Microvascular Endothelial Cell Metabolism Underpins Sex-dependent Vascular Morphogenesis in Lung Development.

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Premature male infants are twice as likely to develop bronchopulmonary dysplasia (BPD) compared to females, in part due to deficits in pulmonary angiogenesis, a process critical for lung alveolarization. The molecular mechanisms behind this sex difference are poorly understood. Endothelial cells predominantly use glycolysis to generate energy but switch to fatty acid oxidation (FAO) when undergoing angiogenesis. Peroxisome proliferator-activated receptor gamma (PPARγ) is a transcription factor that is responsible for upregulating FAO. While PPARγ has been studied as a pathway to target in BPD, no studies have looked at the sex-specific expression of PPARγ or the influence sex hormones play in this pathway. Understanding the role that sex plays in PPARγ is critical for the development of treatment regimens in male and female premature infants. In this study, our objective was to determine the influence of sex and sex hormones, estradiol and dihydrotestosterone, on the expression of PPARγ and their influence on cellular metabolism. Human pulmonary microvascular endothelial cells (HPMECs, 18-19 weeks gestation) were routinely cultured and used in experiments from passages 3-6. Six donors (3 male, 3 female) were used to test sex differences. Cells were dosed with physiological levels of dihydrotestosterone (DHT) or 17-β estradiol (E2) to determine the influence of these sex hormones on growth, metabolism, and protein expression. Overall, female neonatal HPMECs grow faster than male cells. Whereas there is a sex-dependent difference in growth, neither DHT nor E2 influenced male or female cell growth. Female HPMECs have a two-fold higher expression of PPARγ compared to male cells under baseline conditions. Further, male HPMECs have a two-fold higher expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme used in glycolysis. Interestingly, PPARγ levels decrease in a dose-dependent manner in male HPMECs treated with E2, while female HPMEC PPARγ levels remain unchanged. The male HPMEC PPARγ response to E2 is especially important given that circulating E2 levels start to increase exponentially around 20 weeks gestation. Even though male HPMECs have a higher baseline compared to female cells, there is no significant change in male or female global metabolism due to treatment with either E2 or DHT hormones. These data demonstrate the importance

of including the sex and sex hormone status of patients when clinically targeting PPARγ pathways because of these sex-dependent, sex hormone sensitive metabolic elements.

P1646/B784

Imaging Epithelial Cell Biology in Ultrathin AT1 Cells.

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The lung epithelium is home to one of the thinnest cells in the mammalian body - alveolar type 1 (AT1) cells. Individual AT1 cells are >10,000 um^2 in surface area but merely 0.1 um thick, approaching the diffraction limit of light microscopy, such that they were unknown before the advent of electron microscopy. Nevertheless, such ultrathin morphology is essential for gases to passively diffuse across into the underlying vasculature. Furthermore, these AT1 cells are active in signaling and have to be mechanically strong to withstand the cyclic breathing motion, requiring their subcellular machinery to function within a confined space and over a large distance, reminiscent of axonal transport in neurons. We have found a key step in AT1 cell thinning is cell junction remodeling and a key transcriptional control element is YAP/TAZ, components of the mechanosensing Hippo pathway. However, it is unclear how the cellular components, such as cytoskeletons, organelles, and junctions, drive and adapt to the unique AT1 cell morphology. We are pursuing this using expansion microscopy and a novel Cre-dependent cell biology reporter mouse that marks 6 cellular components with distinct colors and is also amenable to real-time imaging. Our work is expected to reveal untapped cell biology unique to large, thin cells and also introduce a general approach to bridge our vast knowledge of in *vitro* cell biology to its in *vivo* counterpart.

P1647/B785

Cell Identity and Differentiation in the Embryonic Pulmonary Mesenchyme.

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In most organs, morphogenesis requires cooperation between epithelial and mesenchymal tissues to pattern changes in tissue shape and cell differentiation. Smooth muscle and smooth muscle-like tissues guide morphogenesis of multiple branched epithelia, including those in the lung, mammary gland and prostate. Despite its importance for airway morphogenesis, it remains unclear how spatiotemporally patterned smooth muscle differentiation is achieved during lung development and whether smooth muscle is recruited from a distinct mesenchymal progenitor population. Here, we carried out single-cell (sc) RNA-Seq of *E*11.5 mouse lungs. Computationally clustering mesenchymal cells showed transitions from undifferentiated mesenchyme to mature smooth muscle, but did not reveal a distinct progenitor population. We found instead that the pulmonary mesenchyme is a heterogeneous but ultimately closely related population of cells. Variability among mesenchymal cells is associated with distinct expression profiles of genes related to Wnt signaling. Manipulating Wnt signaling in lung explants impacts the relative abundance of undifferentiated mesenchymal cells and airway smooth muscle. By examining gene expression along reconstructed differentiation trajectories obtained using diffusion analysis of our scRNA-Seq data, we identified gene sets that fluctuate in similar patterns over the course of smooth muscle differentiation. Enrichment analyses of these gene sets revealed signaling pathways that may be implicated at different stages of differentiation. In particular, we find major changes in cytoskeleton and extracellular matrix-related genes, suggesting a role for mechanical signals in

regulating airway smooth muscle differentiation. We are carrying out motif discovery in DNase-hypersensitive regions near these gene sets to identify candidate transcription factors that may control smooth muscle patterning. Our work provides the first single-cell view of embryonic airway smooth muscle differentiation and sheds light on how physical and biochemical signals are interpreted by the mesenchyme to pattern smooth muscle.

P1648/B786

***Syd/mapk8ip3* Regulates Hippo Signaling to Control Tissue Growth.**

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How organisms control organ size during development is not fully understood. The MAPK scaffolding protein JNK interacting protein 3 (JIP3) has been implicated in organ size regulation in mammals. The underlying mechanisms remain unclear. Depletion of the JIP3 ortholog Sunday Driver (Syd) in the developing wing significantly reduced adult wing size, consistent with mammalian data. It is known that the evolutionarily conserved JNK and Hippo pathways cooperate to control tissue size across species. In *Drosophila*, localized JNK activity modulates Hippo activity to regulate cell proliferation throughout the developing wing, thus controlling adult wing size. Our mechanistic studies revealed that Syd/JIP3 functions downstream of the JNK-Hippo signal relay. Syd/JIP3 inhibition did not interfere with Hippo transcriptional outputs. Instead, Syd/JIP3 is required for the function of DIAP1. Syd/JIP3 inhibition does not affect DIAP1 transcription but it leads to a decrease in DIAP1 protein. This is accompanied by a corresponding increase in cell death and ultimately leads to smaller adult wings. Furthermore, partial restoration of DIAP1 activity using a stabilized *DIAP1* allele was sufficient to rescue *Syd/Jip3* small wing phenotype. Furthermore, we found that tissue size responses to hippo pathway deregulation are sensitive to Syd/JIP3 dosage. Taken together, we conclude that Syd/JIP3 regulates wing size by controlling DIAP1 protein stability, thereby modulating the cellular response to Hippo size control mechanisms. Considering that Hippo and Syd/JIP3 are conserved in flies and mammals, our findings provide potential mechanistic insights explaining the recent and perplexing link between *jip3* mutations and organ size defects in mammals.

P1649/B787

Morphological Characterization of *Small*, *Dumpy*, and *Long* Phenotypes in *Caenorhabditis Elegans*.

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What determines the size of organism is an intriguing question in animal development, but not fully understood, yet. In *Caenorhabditis elegans*, a large number of genes have been identified that regulate the worm's body size as well as morphology. While many collagen genes are known to directly affect the worm size and morphology by molding curticle, several major pathways including TGF-β, RUNX and calcineurin also largely affect body size by controlling cell size and number. Though the body size of *C. elegans* has been recognized as an obvious developmental phenotype, clear and distinct standards have not been established to indicate a worm's appeared size and shape. In this study, we measured the body lengths, body widths, terminal bulb lengths and head sizes of various mutant worms that have small, dumpy or long phenotypes, and plot the ratios of body length/width and of head size/pharyngeal size. We found that mutant worms showing each phenotype of small, dumpy or long tend to cluster, and certain mutants are out of the cluster, indicating that there is a common feature among mutants showing similar phenotype. These results suggest that small phenotypes are proportionally smaller

overall with mild stoutness and dumpy phenotypes are significantly stouter and have disproportionate head sizes. Accordingly, we reassign new body size phenotype to certain mutants, which were previously reported otherwise.

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Reciprocal Regulation between DBL-1/BMP Signaling and Cuticle Collagen Genes.

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Cell differentiation and homeostasis depend on interactions with the cellular environment. In addition to responding to their environments, cells shape them, in part by modulating the extracellular matrix (ECM). Disruption of these interactions results in pathologies such as cancer or fibrosis. Signaling by Transforming Growth Factor-β (TGF-β) family ligands is known to modulate ECM, and TGF-β signaling pathways may respond to matrix stiffness. We have established the nematode *C. elegans* as an in *vivo* model to study the interplay of TGF-β signaling and ECM. *C. elegans* DBL-1, a TGF-β family member related to BMP2/4, is a determinant of body size. We previously showed that DBL-1/BMP signaling determines body size through transcriptional regulation of cuticle collagen genes. The *C. elegans* cuticle is a flexible barrier composed of multiple collagen layers with a lipid-rich epicuticle. We have now obtained evidence of feedback regulation of DBL-1/BMP by collagen genes. Inactivation of DBL-1-regulated cuticle collagen genes reduces DBL-1/BMP signaling, as measured by a Smad activity reporter. Furthermore, depletion of these collagens reduces GFP::DBL-1 fluorescence, and acts unexpectedly at the level of *dbl-1* transcription. We conclude that cuticle, a type of ECM that is functionally analogous to the outermost epidermal barrier in mammalian skin, impinges on DBL-1/BMP expression and signaling. The feedback regulation of DBL-1/BMP signaling by collagens is likely to be contact-independent, due to the physical separation of the cuticle from DBL-1-expressing cells in the ventral nerve cord. We are therefore testing the hypothesis that biomechanical inputs regulate DBL-1/BMP signaling. We used atomic force microscopy (AFM) in intact animals to determine how the structure and mechanics of the cuticle differ in genotypes with cuticle collagen mutations. We analyzed two cuticle collagen mutant backgrounds: *rol-6,* a DBL-1-regulated collagen gene, *and dpy-5,* a control collagen gene. Assuming Derjaguin-Muller-Toporov (DMT) model of elastic contact of the AFM probe tip-sample, the average values of the cuticle stiffness are 0.73 MPa, 0.47 MPa, and 0.56 MPa for wild type, *rol-6*, and *dpy-5*, respectively. Therefore, loss of DBL-1-regulated ROL-6 has a greater effect on cuticle stiffness than loss of DPY-5. We will analyze additional genotypes to determine whether changes in stiffness correlate with changes in DBL-1/BMP signaling. In summary, the DBL-1/BMP pathway provides a unique in *vivo* model to study bidirectional interactions between cell signaling and the ECM in the context of the intact organism. We propose that reciprocal interactions permit robust yet environmentally-responsive control of body size.

P1651/B789

A Genetic 'Brain-body Axis' of Morphogenesis: Neurotransmitter Receptors as Key Regulators of *Drosophila* Epithelial Morphogenesis.

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Organ development relies on a symphony of signals that are coordinated spatiotemporally. Emerging research has implicated the role of neurotransmitter receptors, belonging to the GPCR superfamily, as possible regulators of pattern formation and morphogenesis. However, how these receptors interact amongst themselves and with other complex signaling pathways to regulate organogenesis is still unknown. To address this gap in knowledge, we performed a systematic RNAi-based screen of GPCRs and G-proteins, combined with deep learning based bio-image analysis tools, to characterize cell autonomous roles of these receptors in *Drosophila* wing growth and morphogenesis. A new deep learning-based bioimage analysis platform automated identification of severe phenotypes within a large set of bio-image data consisting of genetically perturbed *Drosophila melanogaster* wings. The machine learning pipeline identified novel neuropeptide GPCRs as regulators of organ growth and morphogenesis in wing development. Machine learning-driven automated feature extraction generated high dimensional phenotypic signatures for each individual wing that was further used to predict protein-protein interactions verified through existing literature and experimental evidence. RT-qPCR and fluorescent labeled gene expression reporters confirmed the predicted, but previously unreported, expression of the identified neurotransmitter receptors in wing imaginal discs. Further, immunohistochemistry assays demonstrated how one of the identified neurotransmitter receptors, 5-HT1B, promotes growth and cell division during wing growth, and severely impacts pupal wing morphogenesis and vein patterning through dysregulation of MAPK signaling. This work elucidates the molecular mechanisms of how multiple neural GPCRs regulate epithelial morphogenesis.

P1652/B790

Altered Cell Cycle Dynamics Enable Whole Organ Regeneration within a Developmental Time Scale in the *Drosophila* Hindgut.

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Development of individual organs is subject to completion within a given time frame, which is set by systemic cues such as hormonal signals. Within this time frame, specific cells and tissues often need to adapt to challenges such as acute injury and cell loss. Since developmental time constraints can limit an organ's regenerative capacity, some injured tissues such as insect imaginal discs can extend developmental timing to allow for organ regeneration. Comparatively, much less is known about how tissues can regenerate an organ without prolonging development. We recently established that the larval *Drosophila* hindgut undergoes whole organ regeneration following acute injury (Cohen et al., 2018). Here, we report that this regenerative response is time-limited and, unlike other *Drosophila* imaginal tissues, does not delay organismal development following injury. Instead, cells of the hindgut pylorus trigger regenerative cell cycles prior to a pulse of the steroid hormone ecdysone. After injury, there is a "race" between these regenerative cycles and developmental ecdysone signaling. At a fixed point in pupal development, ecdysone opens the chromatin landscape within an enhancer of the *fizzy-related* (fzr) gene. We previously found that expression of *fzr,* which has a conserved role in degradation of mitotic cyclins, causes a loss of regenerative potential in favor of non-regenerative hypertrophic cell

cycles. Our results suggest a direct link between developmental cues and limitation of an organ's regenerative capacity. In investigating the mechanism that accomplishes whole organ regeneration before ecdysone-mediated *fzr* expression, we found that pyloric cell cycle dynamics are altered to allow for additional rounds of mitosis upon injury. Cytokines of the JAK-Stat pathway are responsible for driving these additional regenerative cell cycles. Our work demonstrates that the *Drosophila* hindgut is a genetically tractable model to reveal how rapid regeneration can be accomplished during organ development, and will uncover conserved mechanisms of injury repair. Relevant References: Cohen, E., Allen, S. R., Sawyer, J. K., & Fox, D. T. (2018). Fizzy-Related dictates a cell cycle switch during organ repair and tissue growth responses in the Drosophila hindgut. *eLife*, *7*, e38327.

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Can Tualang Or Manuka Honey Prevent the Rate of Development Changes and Malformations Seen After Bisphenol a Exposure in *Xenopus Laevis* (clawed Frog) and *Rana Pipiens* (leopard Frog)?

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Bisphenol a (BPA), is a well-known environmental endocrine disruptor (EED) used in common household items. The negative health effects of BPA exposure include increased cancer risk, infertility, and birth defects. The EED properties of BPA are due to its ability to interfere with estrogen and thyroid hormone action; BPA has also been correlated with an increase in oxidative DNA base lesions. Thottumari et al. (2019) showed that BPA, and its common substitutes, BPS and BPF, individually and in combination, caused malformations in the metamorphosis of *Xenopus laevis* (clawed frog). Wisniew (2009) exposed rats to BPA; those exposed to only BPA developed ovarian cysts, those treated with Tualang honey (TH) did not. Research suggests Manuka honey (MH) shares similar protective properties as TH, due to at least partly the activity of one of its components, kaempferol, a flavonoid. This study was conducted to determine if TH and/or MH can prevent and/or reverse BPA-induced malformations. *X. laevis* specimens were incubated or pre-incubated at Nieuwkoop and Faber tailbud stage 25 (Nieuwkoop and Faber, 1994). *Rana pipiens* (leopard frog) specimens were incubated at Gosner stage 15 (Gosner, 1960). Specimens were studied under these conditions: (1) pre-incubated in honey solution prior to BPA solution at the same concentration, (2) incubated in BPA prior to honey solution of the same concentration, (3) incubated in either BPA or honey solution, and (4) incubated in combination of equal parts BPA and honey solution of the same concentrations. Concentrations used were: 3μ;g/ml BPA, 3μ;g/ml TH, 3μ;g/ml MH, 3μ;g/ml of kaempferol, 5μ;g/ml BPA, 5μ;g/ml TH, 5μ;g/ml MH, and 5μ;g/ml kaempferol. Preliminary results indicate differences in mortality, length, rate of development, and BPA-induced malformations. Specimens pre-incubated in BPA then treated with a honey solution presented less severe malformations in the presence of TH and/or MH. Specimens pre-incubated in a kaempferol solution, specimens incubated in a BPA-kaempferol mixture, and specimens pre-incubated in BPA solution prior to kaempferol displayed the lowest rates of mortality and greatest average growth. Such findings indicate that kaempferol has a possible protective property against BPA exposure. Also, an updated review of the medicinal effects of Tualang honey and Manuka honey was done. This research was funded by grants from the TriBeta Biological Research Foundation and the Independent College Fund of NJ.

P1654/B792

Dchs1 Regulated Mirna Processing and Its Effects on Valve Endocardium Stabilization.

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Mitral valve prolapse (MVP) is one of the most common forms of cardiac valve disease and affects 1 in 40individuals worldwide. MVP can lead to arrhythmias, heart failure, and sudden cardiac death and 1 in 10 patients will require valve surgery. Surgery for MVP is now the fastest growing cardiovascular intervention in the Western world. As such, MVP carries a significant burden of morbidity and mortality. Our lab was the first to identify the cause of non-syndromic MVP using a combination of linkage analyses, as well as exome and capture sequencing to identify loss of function mutations in the cadherin gene, *DCHS1.* Two-hybrid screens were undertaken to further understand Dchs1 function and the RNA binding protein, LIX1L was identified as the only interacting protein. LIX1L binds and promotes miRNA processing through interactions with the microprocessor which is made up of the proteins DROSHA and DGCR8. Expression studies have corroborated this theory as DCHS1, LIX1L, and the microprocessor proteins are expressed in endothelial cells in the mitral valve. Cell culture data shows that a loss of DCHS1 compromises processing of target miRNAs through the microprocessor leading to a significant decrease in pre-miRNA expression. Expression studies have also shown that loss of Dchs1 reduces valve endocardial stability, which may be caused by the loss of miRNAs. These studies illustrate the importance of DCHS1 effects on valve endocardium stabilization in MVP. Uncovering how these changes lead to clinically significant pathology later in life is crucial to the characterization of MVP.

P1655/B793

Cytoneme-mediated Cell-cell Communication Depends on Contact-dependent Synaptic Matchmaking Process Mediated by Receptor-ligand Interactions.

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Cytonemes are actin-based signaling filopodia that are essential for direct cell-cell communication of morphogenetic signaling proteins at the point of cell-cell contact. Although cytonemes are found in both vertebrate and invertebrate tissues and are shown to be involved in most signaling pathways, the mechanistic basis of cytoneme-mediated signaling is still poorly understood. This work uses Drosophilatracheal tissue to address a fundamental question: how cytonemes emanating from the cells might find the right target to establish signaling contacts. Tracheoblast cells of Drosophila larval air-sac primordium, a precursor of adult air-sac, extend long polarized cytonemes containing FGFR to contact a group of FGF expressing cells in the wing imaginal discs and receive FGF directly. By live imaging analyses of the developing larval tissue, we discovered that the FGF-expressing cells also extend actin-based cytonemes containing FGF to reach out to the FGFR-containing ASP cytonemes. Dynamic contacts that the source and recipient cytonemes with each other determine their polarized orientation toward each other. Further investigation revealed that the receptor-ligand interaction is the primary molecular determinant of the dynamic interactions of the signaling cytonemes. We further showed that this mode of communication via signaling contacts leads to a coordinated migration of the source and recipient cells contributing to the branching morphogenesis of the ASP.

P1656/B794

Frizzled 6 Disruption Inhibits Osteoblastic Differentiation Induced by Titanium with Nanotopography through Wnt/β-catenin Signaling Pathway.

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The wingless-related integration site (Wnt) signaling pathways are involved in cell differentiation and homeostasis of adult tissues, including bone. Our group have shown that a unique nanotopography generated on titanium (Ti) surfaces favors osteoblastic differentiation by modulating integrin and bone morphogenetic protein signaling pathways. However, few studies have addressed the role of Wnt on osteoblast response to nanoscale topographies. As both the canonical Wnt/β-catenin and non-canonical Wnt/Ca^{2+} signaling play key roles in osteoblastic differentiation, here we aimed to investigate the participation Wnt signaling in the higher osteogenic potential of Ti with nanotopography (Ti-Nano) and which pathway, Wnt/β-catenin or Wnt/Ca^{2+}, is more relevant to this process. Ti discs were treated with sulphuric acid/hydrogen peroxide solution to produce Ti-Nano and discs without treatment were used as control (Ti-Control). MC3T3-E1 cells were cultured on both surfaces to evaluate the effect of Ti-Nano on the expression of genes related to canonical Wnt/β-catenin and non-canonical Wnt/Ca^{2+} signaling pathways. Based on real-time PCR data, one of the most intensely modulated genes by Ti-Nano, the Wnt/Ca^{2+} pathway-related frizzled 6 (*Fzd6*), was selected and silenced by CRISPR. Then, we investigated the effect of *Fzd6* disruption on osteoblastic differentiation of cells grown on Ti-Nano and Ti-Control. The experiments were repeated at least twice in triplicate (n=3) and the data were compared by Student's t-test or one-way ANOVA ($p \leq 0.05$). The *Fzd6* disruption inhibited osteoblastic differentiation on both Ti surfaces with a more pronounced effect in cells grown on Ti-Nano during early stages of culture development. The analysis of the expression of calcium-calmodulin-dependent protein kinase II (CaMKII) and β-catenin surprisingly demonstrated that the *Fzd6* silencing reduced the osteoblastic differentiation induced by Ti-Nano by preventing the activation of Wnt/β-catenin signaling pathway, but not of Wnt/Ca^{2+}, the most common pathway triggered by Fzd6 receptor. In conclusion, our results shed lights on the biological function of Fzd6 as a gatekeeper that trigger Wnt/β-catenin signaling pathway and in osteoblasts and contribute to the understanding of the cellular mechanisms regulated by nanotopography during osteoblast differentiation. **Grants:** FAPESP (# 2016/14171-0 and 2016/14711-4), CNPq (# 303464/2016-0) and CAPES.

P1657/B795

Assessing the Utility of the Transcription Factor Creb3l1 as a Potential Osteogenic Target Using Crisp/cas9 Induced Truncations in the Zebrafish Model.

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CREB3L1 is a helix-loop-helix transcription factor required for bone formation, and CREB3L1$^{-/-}$ mice exhibit bone defects similar to those seen in osteoporosis patients. Understanding the mechanisms that facilitate bone development and maintenance is crucial to identify targets for preventing or treating osteoporosis. CREB3L1, unlike most transcription factors, is synthesized as a trans-membrane protein and inserted into the ER membrane with a luminal C-terminal (CTer) domain and a cytosolic N-terminal transactivation (TA) domain that regulates the transcription of genes crucial for bone homeostasis. However, we lack an understanding of the role the CTer region plays in bone formation. We use the

Zebrafish (*Danio rerio*) to perform a structure/function analysis of CREB3L1 by engineering CRISP/Cas9-mediated truncations in exon 2 (CREB3L1$^{-/-}$ allele encoding the N-terminal 63 amino acids) and exon 9 (CREB3L1$^{TA/TA}$) encoding the N-terminal 374 amino acids that encompasses the entire TA. Bone development was assessed in CREB3L1$^{-/-}$ embryos by Alizarin red and Alcian Blue staining and in adult fish by histological and µCT analysis. CREB3L1$^{-/-}$ embryos and adult fish appear to have normal development, tissue morphogenesis, and bone formation. We are in the process of analyzing the CREB3L1$^{TA/TA}$ allele and its impact on development and bone homeostasis in zebrafish. To gain insight into the behavior of the fragments produced by the two alleles, we accessed the localization and dynamics of the fragments in HeLa cells. We uncovered that the 63 aa fragment aggregates in the cytoplasm, and most likely is dysfunctional. In contrast, the TA fragment is efficiently translocated into the nucleus, suggesting that it is constitutively functional in transcribing genes involved in bone development. Our studies have the potential to define CREB3L1 as a prospective target to prevent or treat osteoporosis.

P1658/B796

Centrosomes Are Essential for the Differentiation of Lung Progenitor Cells during Branching Morphogenesis.

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Centrosomes are the major microtubule-organizing centers of animal cells that build mitotic spindles during mitosis and axoneme during ciliation. Here we analyzed the roles of centrosome in rapidly dividing mouse endodermal epithelia using Cre under the control of Shh promoter to delete floxed Cenpj allele, which is essential for centriole biogenesis. Centrosomes and cilia were absent from epithelia of the mutant lung. Mutant lungs were much smaller than controls and were defective in branching after major lobes were formed. Lung branching of Epithelia specific IFT88 knockout was indistinguishable from that of controls, indicating that cilia were dispensable for lung branching morphogenesis. To investigate the cell type compositions, I stained the control and mutant lungs for Sox2 and Sox9. The size of mutant lung and the populations of Sox9+ progenitors expanded along the timeline, while no Sox2+ bronchioles were detected, indicating that mutant lung progenitors can't differentiate into Sox2+ bronchioles. RNAseq showed robust upregulation of p21, while a dramatic reduction of Sox2 in the mutant lung. This is consistent with previous studies that p53-p21 pathway suppressed Sox2 expression. Activation of p53 apoptosis pathway was confirmed by Staining of p53, cleaved Casp3 and TUNEL. To test whether Sox2 is the key factor required for normal lung branching, we deleted Sox2 in lung epithelia using Shh-Cre. Surprisingly, branching of the Sox2 conditional knockout mutant is indistinguishable from that of controls, indicating that Sox2 is dispensable for lung branching at the pseudoglandular stage. Then we tested whether p53 dependent apoptosis suppressed the differentiation of Sox9+ progenitor cells and the branching morphogenesis by knocking out p53 in Cenpj conditional knockout mutant. The branching morphogenesis of the mutant lung was successfully rescued by p53 knockout. This indicates that centrosome loss-induced p53 dependent apoptosis blocked the differentiation of Sox9+ progenitor cells and caused the lung branching defect.

Stem Cells-HSCs

P1659/B797

A Role for the Tetraspanin CD82 in Modulating Hematopoietic Stem Cell Quiescence, Activation, and Bone Marrow Niche Localization.

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Hematopoietic stem and progenitor cells (HSPCs) provide the cellular reservoir that gives rise to the highly varied blood and immune cells required to support the lifespan of an organism. Due to high cellular turnover, the hematopoietic system requires an equilibrium between HSC self-renewal, commitment to differentiation, and maintenance of quiescence states in order to preserve a lifelong blood and immune supply. Moreover, tightly orchestrated control of quiescence, self-renewal and differentiation is critical for sustaining the regenerative needs of the hematopoietic system under stress conditions. While normally maintained in a quiescent state, HSPCs can rapidly activate to proliferate and differentiate in response to acute stresses such as the regenerative challenges stimulated following HSPC transplantation. Emerging evidence from our laboratory and others suggests that the tetraspanin CD82 plays a critical role in the regulation of HSPC quiescence and activation. In the present study, we explore how the bone marrow microenvironment contributes to CD82-mediated HSPC quiescence and activation during homeostasis and stress conditions. CD34+ progenitor-like cell lines with differential CD82 expression were generated and injected into NSG mice to evaluate bone marrow distribution and growth in vivo. While control cells appear to grow in site-specific colonies within the bone marrow, CD82KD cells infiltrate the entire marrow cavity, suggesting an augmented bone marrow localization upon CD82KD. Next, we went on to analyze the CD82KO mice, which our lab has previously shown to have a decrease in the number of long-term HSCs and a disruption in their repopulation potential. Complete blood count measurements of wild type (WT) and CD82KO mice suggest that CD82KO mice have an altered number of white blood cells. Moreover, histochemical analysis of long bones suggests that CD82KO mice display aberrant megakaryocytes. As a measure of stress-induced HSPC activation, serial transplantation studies were completed with isolated WT and CD82KO HSPCs. An alysis of primary and secondary transplants indicates that both WT and CD82KO HSPCs engraft and expand the blood and immune cell populations similarly. Interestingly, at the tertiary transplant, WT HSPCs begin to show normal signs of exhaustion, however, in contrast, CD82KO HSPCs appear to stabilize. At the quaternary transplant, the WT HSPCs continued toward exhaustion, whereas the CD82KO HSPCs display a myeloid-specific cell expansion. These data suggest CD82 regulates HSPC microenvironment interactions. Future studies will be directed at determine the mechanism by which CD82 drives HSPC localization to specific bone marrow sites and how this localization impacts homeostatic and stress-induced expansion.

P1660/B798

Syndecan-2: a Novel Marker and Regulator of Hematopoietic Stem Cells.

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Hematopoietic stem cells (HSCs) maintain the hematopoietic needs of an individual during their lifetime through the careful balance of self-renewal and differentiation. Syndecans are heparan sulfate proteoglycans which regulate cell adhesion, proliferation, and survival. While Syndecans contribute to neural, muscle, and endothelial stem and progenitor cell maintenance, the role of Syndecans in

regulating HSCs is not well understood. To address this, we isolated primary mouse bone marrow (BM) long-term HSCs ($Lin^-/c\text{-}Kit^+/Sca\text{-}1^+/CD34^-/CD48^-/CD150^+$) and lineage negative ($Lin^-$) hematopoietic progenitor cells to quantify Syndecan gene expression. Mouse BM HSCs express fourteenfold increased levels of *Sdc2* compared to Lin^- cells. These findings are further supported by a tenfold increase in Syndecan-2 surface expression on long-term HSCs compared to Lin^- cells, while we do not find increased Syndecan-1, 3 or 4 surface expression on long-term HSCs compared to Lin^- cells. To evaluate the contribution of Syndecan-2 surface expression to hematopoietic differentiation and HSC self-renewal, we performed competitive HSC transplantation assays using fluorescence activated cell sorting to isolate Syndecan-2^+ or Syndecan-2^- HSCs ($Lin^-/c\text{-}Kit^+/Sca\text{-}1^+/CD34^-$). Mice transplanted with Syndecan-2^+ HSCs exhibit a tenfold increase in multi-lineage (B, T, myeloid, and erythroid cell) hematopoietic reconstitution in the blood and bone marrow in primary and secondary transplants compared to mice transplanted with the identical dose of Syndecan-2^- HSCs or unsorted HSCs. Taken together, these data indicate that Syndecan-2 expression can be utilized to isolate HSCs with increased hematopoietic differentiation potential and self-renewal capacity. To test the functional role of Syndecan-2 in regulating HSCs, we performed competitive transplant assays using *Sdc2*$^{-/-}$ knockout mice and littermate *Sdc2*$^{+/+}$ mice. Mice transplanted with BM cells from *Sdc2*$^{-/-}$ donors display significantly decreased multi-lineage (B, T, myeloid, and erythroid cell) hematopoietic engraftment compared to mice transplanted with the identical dose of BM from *SDC2*$^{+/+}$ donors. These data suggest that Syndecan-2 expression is critical for HSC self-renewal in *vivo*. Mechanistically, we find a significant increase in the proportion of apoptotic *Sdc2*$^{-/-}$ HSCs compared to *Sdc2*$^{+/+}$ HSCs, indicating that Syndecan-2 regulates HSCs by controlling cell survival signaling. Collectively, our results suggest that Syndecan-2 is a novel marker for HSCs and has an essential role in regulating HSC differentiation and self-renewal in *vivo*, which has broad applications to the field of stem cell biology.

P1661/B799

Quiescence/mobilization of Hematopoietic Stem Cells Induced by Polyphenols from Green Tea through APC/EPCR/PAR-1 Axis.

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Hematopoietic stem cells (HSC) mobilization is induced by thrombin through coagulation thrombin/PAR-1 axis, and quiescence is maintained across the APC/EPCR/PAR-1 axis. Our goal was to investigate the effect of green tea polyphenols (GT) on thrombin/PAR-1 and APC/EPCR/PAR-1 axis. GT extract (250 mg/kg body weight) was given orally (gavage) once every 7 days to mice (n=6) injected i.p. with 100µg lipopolysaccharide (LPS). Controls received vehicle only. After 24h, mice were euthanized; bone marrow (BM) and peripheral blood (PB) were collected for the assays. LPS was chosen because it induces thrombin release in BM. GT partially prevented the reduced EPCR expression in BM HSC (LSK: $Lin^-Sca^+c\text{-}kit^+$) of LPS-injected mice, but did not affect the increased PAR-1 expression in circulating HSC and mature cells. Evans blue analysis revealed a reduction in the vascular permeability of the BM of LPS-injected mice treated with GT. To address whether GT altered NO production, we assayed NO levels and eNOS phosphorylation in immature LT-HSC (LSK $EPCR^{high}$ cells). NO production is activated by eNOS phosphorylation at Ser1177 and negatively regulated by eNOS phosphorylation at Thr495. GT reduced BM LSK $EPCR^{high}$ percentage with higher intracellular NO induced by LPS and increased eNOS phosphorylation at Thr495 in these cells. In order to evaluate the effect of GT on the functional ability of HSC, we performed a BM competitive repopulation assay. Donor mice (C57BL/6J) received or not GT

followed by LPS injection (groups: GT+LPS and LPS). BM donor cells were transplanted (1:1) into lethally irradiated receptors (B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ) together with BM cells from competitors (B6.SJL-Ptprc^{a}Pepc^{b}/BoyJ). Mice were followed for 16 weeks and hematological analysis revealed no difference in circulating leukocytes, platelets, or hemoglobin levels. A higher percentage of donor cells were found in PB of GT+LPS group compared to LPS. We also observed an increase of circulating T lymphocytes and myeloid cells. After 16 weeks, recipients were euthanized. LSK and LSK EPCR^{high} populations were increased in BM of GT+LPS group compared to LPS, although only LSK EPCR^{high} percentage was statistically different. Taken together, our results demonstrate that GT increased the functional ability of HSC showing increased percentage of bone marrow LSK EPCR^{high} (or LT-HSC), which are the most quiescent stem cells with strong self-renewal ability. Furthermore, GT prevented EPCR expression and NO production induced by LPS in immature cells, displaying an anti-inflammatory effect that leads to the maintenance of barrier integrity and quiescence, which was corroborated by reducing BM vascular permeability. Thus, GT appears to modulate quiescence/mobilization of immature HSC through APC/EPCR/PAR-1 axis.

P1662/B800

Exploring the Role of the Notch Signalling Pathway in Human Haematopoietic Stem and Progenitor Cells.

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Notch is an evolutionarily conserved signalling pathway with roles in stem cell maintenance, survival and differentiation. While studies in the murine haematopoietic system have proposed different roles for Notch signalling under stress and steady-state conditions, whether this is involved in the regulation of human haematopoietic stem/progenitor cells (HSPCs) has been scarcely explored. Here, we employed not only pharmacological inhibition by using the γ-secretase inhibitor DAPT but most importantly using shRNA-mediated silencing of Notch pathway members to evaluate the potential functions of Notch may have in the regulation of human HSPC biology. Immunodeficient (NSG) mice were transplanted with human CD34^{+}CD38^{-} HSPCs. Six-to-seven weeks later, DAPT or vehicle was administered to different cohorts of animals, and at twelve weeks post-transplantation the bone marrows of the recipients were analysed. Notch inhibition caused a significant reduction in human engraftment in mice and deregulation of most stem (HSC) and progenitor cell fractions. Specifically, the HSC population was reduced by 7-fold when compared to the control cohort. Using limiting cell dose assay in secondary transplantation to evaluate the self-renewal capacity of HSPCs, we observed a 2-fold reduction in the secondary repopulating frequency from HSPCs that had been treated with DAPT. To address whether these effects were caused by cell-autonomous loss of Notch signalling, HSPCs were transduced with shRNAs targeting the *NCSTN* or *RBPJ* genes. *NCSTN* encodes for the protease Nicastrin (a component of the γ-secretase complex), the loss of which blocks the processing of Notch receptors. Silencing *RBPJ* (encoding for Notch co-activator RBPJκ) specifically inhibits canonical Notch signalling. Both the loss of Nicastrin and RBPJκ resulted in a significant loss of primitive cells in *vitro*. Transplanting *NCSTN* or *RBPJ*-silenced HSPCs into NSG mice led to a reduction in human engraftment at 12 weeks. While a 3-fold reduction was observed in the sh*NCSTN* cohort, a dramatic 68-fold decrease in sh*RBPJ*-transplanted animals was noted. In agreement with our previous observations, the HSC compartment was significantly reduced in both conditions when compared to the control cohort. A trending increase in downstream progenitors suggests enforced differentiation from the HSCs compartment. Furthermore,

Notch inhibition led to an overall shift toward myeloid cell differentiation at the expense of the lymphoid fraction - an effect that was specific to RBPJκ loss. Altogether, these results support that blocking Notch signalling impaired human HSCs in *vivo in* a cell-autonomous manner *and* differ from studies that support little role of this pathway in regulating murine HSPCs.

P1663/B801

Microtubules Deform the Nucleus and Force Chromatin Reorganization during Early Differentiation of Human Hematopoietic Stem Cells.

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Hematopoietic Stem Cells (HSC) can differentiate into all hematopoietic lineages in order to support hematopoiesis. Cells from the myeloid and lymphoid lineages fulfill distinct functions with specific shapes and intra-cellular architectures. The role of cytokines in the regulation of HSC differentiation has been intensively studied but our understanding of the potential contribution of inner cell architecture is relatively poor. Here we show that large invaginations are generated by microtubule constrains on the swelling nucleus of human HSCs during early commitment toward the myeloid lineage. These invaginations are associated with chromatin reorganisation, local loss of histone methylation and changes in expression of specific hematopoietic genes. This establishes the role of microtubules in defining the unique lobulated nuclear shape observed in myeloid progenitor cells and suggests that this shape is important to establish the gene expression profile specific to this hematopoietic lineage. It opens new perspectives on the implications of microtubule-generated forces, in the early specification of the myeloid lineage.

P1664/B802

The Role of Galectins and *O*-GlcNAc in Regulating Promyelocytic Cell Stemness and Differentiation.

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Cell differentiation is associated with multiple changes in gene and protein expression profiles, and specific post-translational modification of regulatory proteins. Our recent studies with promyelocytic HL-60 stem-like progenitor cells demonstrate that neutrophilic differentiation leads to significant down-regulation of global *O*-GlcNAcylation of intracellular proteins and changes in expression of galectins, soluble beta-galactoside-binding proteins with diverse glycan-dependent and glycan-independent functions outside and inside cells. Molecular mechanisms controlling secretion of galectins remain poorly understood since these proteins do not have an ER-targeting N-terminal signal sequence or hydrophobic segment and therefore use unconventional transport pathways. Bioinformatics analysis reveals that galectins have sites for *O*-GlcNAcylation, which potentially can control protein trafficking and distribution between intracellular compartments. Considering these findings, we elaborate the novel hypothesis that regulatory functions of galectins toward self-renewal and differentiation of promyeloid cells depends on the localization of galectins in cells which is driven by *O*-GlcNAc signaling mechanisms. In particular, we propose that high levels of *O*-GlcNAc promote intracellular accumulation of galectins in stem cells while low levels of *O*-GlcNAc promote galectin secretion and cell differentiation. This concept and results of our on-going experiments will be presented. Supported by NSERC Discovery Grant (RGPIN-2019-06628).

Cell Fate Determination 1

P1665/B803

Revealing the Function of the E3 Ubiquitin Ligase Cullin-9 in Neural Stem Cell Differentiation.

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The cullin-ring ligase (CRL) family of E3 ubiquitin ligases plays a critical role in the regulation of early developmental processes, including synapse formation and axon guidance. However, Cullin-9 (CUL9) has proven to be a unique CRL with an elusive function. CRLs generally form large complexes that ubiquitinate a set of specific substrates. CUL9 has not been shown to form large complexes and has only two identified substrates. My data suggest a role for CUL9 in the early neural induction phase of cortical differentiation. CUL9 protein levels are induced during cortical glutamatergic differentiation. Interestingly, differentiation of CUL9 knockout (KO) human pluripotent stem cells (hPSCs) to neural progenitor cells (hNPCs) and early born neurons results in inconstant expression of neural-specific markers. Additionally, CUL9 KO hNPCs form smaller embryoid bodies and neural rosettes with variable lumenal areas. This finding suggests that hNPCs produced from CUL9 depleted hPSCs may have aberrant differentiation potential or self-renewal capacity. We hypothesize that CUL9 may be critical for proper neural rosette formation and subsequent neurogenesis through its regulation of substrates key for neural cell fate. Our main goal is to identify the CUL9 substrates critical for proper neural rosette formation. Previous mass spectrometry analysis of CUL9 immunoprecipitation from hPSCs and hNPCs have identified the cell cycle regulator the anaphase-promoting complex/cyclosome (APC/C) as a potential negative regulator of CUL9 in hPSCs. We are currently performing a quantitative proteomics screen using Isobaric Tags for Relative and Absolute Quantification (iTRAQ) in hPSC, hNPC and cortical neuron CUL9 KO cells to identify proteins enriched in the absence of CUL9. Proteins identified by iTRAQ will be screened as potential CUL9 substrates. I anticipate that elucidating the function of CUL9 in neural induction will provide key insight into the underlying mechanisms linking ubiquitin-mediated regulation in control of neural cell fate, neural rosette formation, and cortical differentiation, setting a framework for studying CUL9's potential involvement in neurodevelopmental disorders.

P1666/B804

Dynamics and Lineage Fate of Proteolipid Protein (plp) Promoter-expressing Cells in the Central Nervous System.

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The proteolipid protein (PLP) is the most abundant protein in the myelin sheath and the Plp gene is highly expressed in oligodendrocytes that form myelin in the central nervous system. Previous studies suggest that Plp gene is expressed in neural progenitors cells much earlier prior to the generation of oligodendrocytes. However, the identity and the fate of Plp promoter-expressing cells are still elusive. We used genetic approaches to permanently label Plp promoter-expressing cells with the reporter gene

enhanced yellow fluorescence protein (EYFP) and employed multi-colored immunohistochemistry to characterize their identity and their lineage fate. We found that Plp promoter-expressing cells were distributed evenly in the embryonic spinal cord and gradually restricted to the ventral domains in the embryonic spinal cord. We showed that, during early neural development stages, Plp promoter-expressing cells were multi-potent neural progenitor cells that gave rise to not only neurons but also glial cells whereas Plp promoter-expressing cells during the late neural development stages only gave rise to glial cells including astrocytes, oligodendrocytes, and ependymal cells. Intriguingly, astrocytes generated from Plp promoter-expressing cells were located only in the ventral spinal cord. Taken together, our study reveals that Plp promoter-expressing cells display a dynamic distribution during neural development and have a broader lineage fate during neural development than oligodendroglial lineage.

P1667/B805

Profiling Genes Regulated by the Neural Crest-Essential Lysine Methyltransferase NSD3 in the Premigratory Neural Crest.

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The neural crest is a transient, stem cell-like population in vertebrate embryos that migrate extensively during development and form a variety of derivatives, including craniofacial skeleton, melanocytes, and the majority of the peripheral nervous system. In our previous work, we identified NSD3 as the first lysine methyltransferase required for proper neural crest specification and migration. While we demonstrated that NSD3 is essential for expression of several key neural crest transcription factors, the extent of gene expression regulated by NSD3 was unknown. To identify NSD3-dependent changes in the transcriptome of premigratory neural crest cells, we performed RNA-seq analysis of dorsal neural tubes isolated from chick embryos electroporated with NSD3 or mismatch control morpholinos. We identified 769 genes with significant changes in expression levels when comparing NSD3 knockdown and mismatch control samples. Using gene ontology analysis, we found groupings of downregulated genes involved in neurogenesis, neuronal differentiation and regulation of cell differentiation, while genes involved in RNA processing and ribosome biogenesis were upregulated when NSD3 was knocked down. To verify the RNA-seq expression level changes, we performed in situ hybridization for select genes of interest in chick embryos electroporated with NSD3 or mismatch control morpholinos. For example, when NSD3 was knocked down in neural folds, we confirmed that the adhesion molecule *astrotactin 1* was downregulated, while the actin binding molecule *tropomyosin 1* was upregulated. From this work, we have identified a collection of putative targets of NSD3 function and potential novel regulators of neural crest development that will be the focus of future studies.

P1668/B806

The Role of Sox11 during Development and Disease: Insights from Coffin Siris Syndrome.

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The development of the complex vertebrate central nervous system (CNS) is tightly regulated to ensure correct temporal and spatial formation of neurons and glia. Progression from neural stem cell to neuron is controlled by the sequential expression of a hierarchy of transcription factors and Sox11, a member of the large Sox HMG transcription factor family is essential in this process. *Sox11* has been shown to play

roles in neuronal differentiation and maturation in a variety of vertebrates. Additionally, our studies in *X. laevis* demonstrate that Sox11 is also involved in the promotion or maintenance of neural stem cells. Studies that examine the loss of Sox11 function demonstrate that it is required for CNS development and mutations in the HMG domain of Sox11 are associated with Coffin Siris Syndrome (CSS). CSS patients have microcephaly associated with reduced cortical tissue and **our results show that these mutations lead to altered CNS development in frog. To** determine how Sox11 plays all these different roles, we identified Sox11 targets during neural cell fate specification (blastula stage) and later at the onset of neurogenesis (mid-gastrula stage). **Our RNA-Seq data confirms that Sox11 is involved early in neural specification and later in development in neural differentiation.** Since Sox11 specificity is determined by protein interactions, we will identify novel partner proteins and Sox11 consensus binding elements, to test the hypothesis that Sox11 is regulating different downstream targets at different times in neural development due to its interaction with different pools of protein partners. With these partners, we will test whether altered partner protein interactions are the cause of the CSS-related phenotype.

P1669/B807

Notch-mediated Polarity Decisions in Lateral Line Hair Cells.

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The development of mechanosensory epithelia, such as those of the auditory and vestibular systems, results in the precise orientation of mechanosensory hair cells and consequently directional sensitivity. After division of a precursor cell in the zebrafish's lateral line, the daughter hair cells differentiate with opposite mechanical sensitivity. This process produces neuromasts containing equal numbers of hair cells of two opposite polarities, half of them sensitive to caudad water movement and half to rostrad flow. Through a combination of theoretical and experimental approaches, we show that Notch1a-mediated lateral inhibition produces a bistable switch that reliably gives rise to hair cell pairs of opposite polarity. Using our mathematical model of the process, we predict the outcome of several genetic and chemical alterations to the system, which we then confirm experimentally. Following the predictions of our model, we are able to alter the ratio of rostrad to caudad cells in the neuromast by titrating the concentration of different inhibitors of the Notch pathway. We confirm these results by generating a transgenic fish line that constitutively expresses the Notch intracellular domain in hair cells, and also by analyzing Notch1a mutant fish. We then show that Notch1a downregulates the expression of Emx2, a transcription factor known to be involved in polarity specification, and acts in parallel with the planar-cell-polarity system to determine the orientation of hair bundles. By analyzing the effect of simultaneous genetic perturbations to Notch1a and Emx2 we infer that the gene-regulatory network determining cell polarity includes undiscovered polarity effectors.

P1670/B808

Doublesex-F as a Novel Cofactor of Abdominal-B in Promoting Female Specific Apoptosis in *Drosophila* Central Nervous System.

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Highly conserved DM domain containing transcription factors (Doublesex/MAB-3/DMRT1) are responsible for generating sexually dimorphic features. In *Drosophila* CNS a set of Doublesex (Dsx) expressing neuroblasts undergo apoptosis in females while their male counterparts proliferate and give

rise to serotonergic neurons crucial for adult mating behaviour. Our study demonstrates that female specific isoform of Doublesex collaborates with Hox gene Abdominal-B (AbdB) to bring about this apoptosis. Biochemical results suggest AbdB and Dsx interact through their highly conserved Homeodomain and DM domains respectively. This interaction is translated into a cooperative binding of the two proteins (AbdB and Dsx) on the apoptotic enhancer in case of females but not in case of males, resulting in female specific activation of apoptotic genes. The capacity of AbdB to utilize sex specific isoform of Dsx as a cofactor underlines the possibility that two classes of proteins are capable of cooperating in selection and regulation of target genes in tissue and sex specific manner. We propose that this interaction could be a common theme in generating sexual dimorphism in different tissues across different species.

P1671/B809

Mutations in the Cholesterol Synthesis Pathway Reveal Independent Functions for Cholesterol and Isoprenoids during Erythropoiesis.

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Erythropoiesis is a tightly regulated and conserved process by which new red blood cells (RBCs) are produced. Defects in erythropoiesis lead to human disorders such as anemia and acute megakaryocytic leukemia. Erythropoiesis is divided into two distinct waves, producing primitive or definitive RBCs. Several essential players that drive erythropoiesis, including the transcription factors GATA1 and FOG, have been previously characterized. We demonstrated previously that the cholesterol synthesis pathway (CSP) is a regulator of erythropoiesis. However, the mechanisms underlying such phenotypes have not been completely elucidated. Therefore, the goal of this study is to determine the mechanism by which CSP regulates RBC differentiation during primitive erythropoiesis. Using a zebrafish transgenic line harboring a mutation in the *hmgcs1* gene, which encodes the enzyme above the rate-limiting step of the CSP, we analyzed RBC differentiation and total number of RBCs. Our results show that mutations in *hmgcs1* led to a decrease of mature RBCs. Further analysis revealed decreased expression of GATA1. Mutations in *hmgcs1* lead to an inhibition of cholesterol and isoprenoid synthesis. Therefore, we used pharmaceutical inhibition to block the synthesis of each individual product. Using this approach, we demonstrate that both cholesterol and isoprenoids regulate RBC development, albeit by distinct cellular mechanisms. Taken together, these results provide evidence of two new upstream regulators of primitive erythropoiesis.

P1672/B810

Interactome an alysis to Study the Molecular Mechanism of Heparin-induced Cardiomyocyte Differentiation.

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Human embryonic stem cells (hESCs) are a powerful model to understand the molecular mechanisms involved in human embryogenesis. Heparin and albumin are common cell culture components, and they are widely used in various cell type-specific differentiation from hESCs. However, their molecular mechanisms in differentiation are often unclear, because it is difficult to identify the functional target among their numerous binding partners. Both heparin and albumin positively promote cardiomyocyte differentiation, and heparin can even generate cardiomyocytes in the absence of WNT inhibitors. Through interactome analysis, we identified a set of binding or functional partners shared by albumin

and heparin from hundreds of candidates. Further analysis identified novel target proteins involved in cardiomyocyte determination. This study highlights interactome analysis as an effective method to study the molecular mechanisms of complicated proteins, and we also revealed new mechanisms involved in cardiomyocyte differentiation.

P1673/B811

Osteogenic Effect of Bmp-9 on Bone Marrow and Adipose Tissue Mesenchymal Stem Cells.

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The cell therapy for bone repair using mesenchymal stem cells (MSC) from bone marrow (BMMSC) or adipose tissue (ATMSC), at least in part, relies on their osteoblast differentiation that can be stimulated by bone morphogenetic proteins (BMP). Within the BMP family, BMP-9 could be a good choice thanks to its high osteogenic potential. The aim of this study was to evaluate the dose-dependent effect of BMP-9 on osteoblast differentiation of BMMSC and ATMSC. Rat BMMSC harvested from femurs and ATMSC harvested from inguinal adipose tissue were cultured under non-osteogenic condition. The media was not supplemented with BMP-9 (Control-C) or supplemented with 25, 50 or 100 ng/mL of BMP-9. The gene expression of the bone markers *Runx2*, alkaline phosphatase (*Alp*) and osteocalcin (*Oc*), and ALP activity were evaluated on day 7 and the mineralized matrix deposition, on day 17. Data were compared by one-way ANOVA and Tukey's test ($p \leq 0.05$). The expression of all evaluated genes was increased by BMP-9 in both BMMSC (*Runx2:* C=50<25<100; *Alp*: C<25<50<100; *Oc*: C=25=50<100) and ATMSC (Runx2: C<50<25=100; *Alp*: C<25<50<100; *Oc*: C<25=50=100) as well as ALP activity BMMSC (C=25=50<100) and ATMSC (C=25<50=100). The mineralized matrix deposition was not affected by BMP-9 in BMMSC but it was increased in ATMSC (C<25=50=100). Despite of not having a dose-dependent effect, BMP-9 showed osteogenic potential and it seems that the more pronounced effects were reached by 100ng/mL. Altogether, these results show that even under non-osteogenic condition BMP-9 was capable of differentiating MSC into osteoblasts and therefore could be useful in MSC-based therapy to regenerate bone tissue.

P1674/B812

Differential Role of Histone Deacetylases in the Differentiation of Chick Embryo Wing Bud Mesenchymal Cells.

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Endochondral bone formation is a complex developmental process involving the differentiation of mesenchymal cells to osteoblasts and well regulated by varieties of factors. Histone deacetylase (HDAC) has been known to be implicated in the differentiation of mesenchymal cells. In this study, we aimed to identify the HDACs and their substrates that are involved in the different stage of differentiation of mesenchymal cells in micromass culture. Pan-HDAC inhibitor trichostatin a (TSA) and class I HDAC inhibitor UF010 suppressed chondrogenesis evidenced by PNA staining, Alcian blue staining, and western blot assay for the expression of type II collagen whereas HDAC4/5 inhibitor LMK-235 and HDAC6 inhibitor showed weaker or little effect on chondrogenesis. While TSA and UF010 significantly enhanced acetylation of histone H3 and TSA, LMK-235, and HDAC6 inhibitor increased acetylation of Runx2. In addition, inhibition of chondrogenesis and cell aggregation by TSA and UF010 was most effective when they were treated for the first two days of culture. Presence of LMK-235 and HDAC6

inhibitor for 10 days promoted osteoblast differentiation evidenced by Alizarin red staining and osteopontin expression. LMK-235 and HDAC6 inhibitor also increased acetylation of Runx2 in day 10 cultured cells. However, they did not affect cell aggregation. The present study show that class I and class II HDACs deacetylate histone H3 and Runx2, respectively and suggest that they play different roles in the differentiation of mesenchymal cells.

P1675/B813

Molecular Characterisation of an Epigenetic Regulatory Circuit Comprising Smad 3 and Ezh2 in an Ipsc-derived Model of Retinal Degeneration.

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Cell fate decisions underpin lineage specification during development and cell differentiation in disease, yet these decisions remain a relatively poorly understood area of cell biology. A core regulator of these events is the Polycomb Repressive Complex 2 (PRC2), which di- and tri-methylates histone 3 at lysine 27 to regulate gene transcription and chromatin organisation, yet little is known about how PRC2 is guided to target specific areas of the genome. Previously in our lab, we identified a functional interaction between Smad3 and EzH2, the catalytic component of PRC2, in multiple cellular contexts. Here we explore this interaction using an induced pluripotent stem cell (IPSC)-derived Retinal Pigmented Epithelium (IRPE) model of TGF-β induced cell fate transition. WTSIi028-A IPSCs, obtained from the European Bank for induced pluripotent Stem Cells, were directed to RPE over 14 days by treatment with combinations of growth factors and small molecule inhibitors including Nicotinamide, Noggin, Dkk-1, IGF-1, bFGF, Activin A, SU5402 and CHIR99021. iRPEs were characterised by RT-PCR and immunocytochemistry for the expression of retinal specific markers. Cell differentiation was induced by treatment with TGF-β 2 for 7 days. RNA-seq was then performed on the BGISEQ platform. Cells were also treated with drugs targeting both TGF-β signalling (SB43152) and EzH2 (GSK343, DzNEP) during this differentiation to further inform signalling events during this process. An alysis of the RNAseq data showed that upon TGF-β treatment, these cells activated a program of extracellular matrix remodelling, promotion of angiogenesis and complement activation, resulting in upregulation of genes such as IGF1, MFAP4, TGFβ1, ECM1 and C1QL1 and downregulation of genes such as CLDN2, AIPL1 and CD46. Further interrogation of the differentially expressed genes with the ENCODE-BROAD institute EzH2 CHIP-Seq dataset in H1 embryonic stem cells (ESCs)(GSM831028) found enrichment of genes with EzH2-bound promotors in ESCs in the set of genes repressed in our cells in response to TGF-β when compared with the upregulated genes. Pharmacological inhibition of EzH2 activity also appears to mitigate some of these TGF-β-induced changes in these cells. The data presented here shows a clear link between TGF-β /Smad3 signalling and EzH2 mediated gene repression in the TGF-β -induced differentiation of iRPE cells which may lead to an exploitable target in certain forms of retinal degeneration.

P1676/B814

TGFβ Signals to Chromatin Via Complexing Interaction of Smad3 with the Polycomb Repressive Complex during the Determination of Renal Epithelial Fate.

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Critical pathological features of diabetic nephropathy are now accepted to include dysregulation of epigenetic processes as evidenced by the observed differential methylation in patients with or without progressive disease. TGFβ resides at the centre of therapeutic approaches for the treatment of renal fibrosis, but few intervention studies have demonstrated clinical efficacy. We recently demonstrated a novel direct interaction between Smad3 and EZH2, the enzymatic component of the polycomb repressive complex 2 (PRC2) during cell fate specification. Targeting the interaction between Smad3 and EZH2 in iPSC derived renal organoids protected against TGFβ mediated tubular epithelial dedifferentiation. Here, we delineate the molecular mechanism underlying this interaction. We have performed ChIP-seq using validated antibodies for Smad3 and EZH2 at specific time points during the derivation of $Six2^{+ve}$nephron progenitors, from iPSCs following the ENCODE Consortium guidelines with optimisation on the Illumina HiSeq platform. Using this genome wide approach, we identified a number of regions throughout the genome that are co-occupied by Smad3 and EZH2 in both undifferentiated iPSCs and during in *vitro* nephrogenesis; ~69% of Smad3 peaks overlapped with EZH2 bound regions in iPSCs and ~32% in the $Six2^{+ve}$nephron progenitor population. Perhaps most significantly, *de novo* motif analysis identified enrichment of the GAGA motif at sites co-occupied by Smad3 and EZH2. This motif was first identified as a Polycomb Response Element (PRE) in Drosophila Melanogaster and subsequently as a region enriched for Mothers Against Decapentaplegic (MAD), the drosophila homolog of mammalian Smads. To the best of our knowledge this is the first description of an analogous PRE in vertebrates. As predicted from our in *silico* analysis, Smad3 and EZH2 were almost exclusively identified at enhancers and superenhancers. An alysis of putative superenhancer target genes by real-time PCR indicated a TGFβ dependent downregulation suggesting a novel mechanism of gene repression during both development and disease. We propose that this complex forms a molecular switch that regulates enhancer and/or promoter access through epigenetic mechanisms and controls gene silencing, informing the fundamental mechanisms through which subsets of genes are switched on and off during fate specification and during the pathogenesis of diabetic nephropathy.

P1677/B815

Growth and Differentiation of Induced Pluripotent Stem Cell-Derived Kidney Organoids Using Fully Synthetic Peptide Hydrogels.

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Induced pluripotent stem cell (iPSC)-derived kidney organoids have been shown to closely recapitulate fundamental tissue developmental processes and represent a useful method to examine aberrant gene regulatory mechanisms that occur in diseases such as diabetic nephropathy. Despite significant progress

made towards understanding signalling mechanisms and refining organoid models over the last number of years, there is a growing necessity to increase complexity, reduce variability and improve maturity and genetic authenticity. We propose that organoid development will benefit from 'tunability' of the biophysical properties of the cellular microenvironment. Synthetic hydrogel scaffolds represent a promising alternative to circumvent limitations associated with tissues grown on traditional, naturally-derived extracellular matrices. These scaffolds are also more amenable to generating tuneable mechanical and signalling gradients that more accurately mimic the in *vivo* environment. We differentiated iPSC-derived nephron progenitors into kidney organoids within fully defined, self-assembling peptide hydrogel scaffolds of variable stiffness and component peptide charges. Transmission electron microscopy of these hydrogels revealed a fibrous structural architecture similar to that of matrigel like-extracellular matrices. Haematoxylin and Eosin staining of encapsulated organoids exhibited variable morphological self-organisation of epithelial cell tubular structures within hydrogels by day 24, particularly between highly charged peptide hydrogels with Young's Moduli of 1 and 14 kPa. Interestingly, immunofluorescent characterisation also showed differential proximal (LTL^{+ve}) and tubular epithelial ($ZO\text{-}1^{+ve}$, $ECAD^{+ve}$) expression between the embedded organoids. Most notably, increased proximal tubule (LTL^{+ve}) formation was observed within softer peptide hydrogels (1 kPa). Additionally, we have identified distinct combinations of extracellular matrix components, including vitronectin and laminin-511, that support differentiation of iPSCs towards renal lineages in two-dimensional culture. Cellular self-organisation into $LTL^{+ve}$and $ZO\text{-}1^{+ve}$ structures accompanied by laminin basement membrane formation was observed by day 18 of differentiation. Functionalisation of these substrates within peptide hydrogels will likely further enhance processes of renal differentiation. To the best of our knowledge, this is the first investigation of iPSC-derived kidney organoids within fully synthetic, self-assembling peptide hydrogels. These results will further support the use of designer matrices that will improve iPSC differentiation towards renal cell fate trajectories.

P1678/B816

Intracellular Ph Dynamics Regulates Intestinal Stem Cell Differentiation.

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Emerging evidence is revealing that intracellular pH (pHi) dynamics regulates different types of epithelial plasticity, including stem cell differentiation, epithelial-mesenchymal transition, and epithelial transformation. In testing the centrality of pHi dynamics in stem cell differentiation we found that increased pHi is necessary for intestinal stem cell (ISC) differentiation, using the well-established 3D *ex vivo* model of adult mouse small intestinal organoids. Using cells expressing the genetically encoded pHi biosensor pHluorin, we identified a pHi gradient along the crypt-villus axis, from lower pHi in crypt cells that include ISCs to higher pHi in differentiated villus cells. Dissipating this gradient and decreasing pHi by inhibiting H+ efflux by the plasma membrane Na-H exchanger NHE1 blocks two stages of organoid development: crypt budding and differentiation to the secretory cell linage. Inhibiting NHE1 activity completely blocks crypt budding over three days despite no effect on polarized expression of the crypt marker CD44. However, increased phosphorylation of myosin light chain 2 (pMLC) seen in controls is lost, which supports a role for actinomyosin contractility regulating budding. Additionally, inhibiting NHE1 and lowering pHi after crypt budding reduces the number of daughter cells expressing ATOH1, a master transcription factor for cells in the secretory lineage, as indicated by $ATOH1^{+}$cell-lineage tracing. Our data reveal a previously unrecognized critical role for pHi dynamics in intestinal epithelial

development and further support an emerging view of pHi dynamics being a conserved regulator of stem cell differentiation.

P1538/B817

The Midbody Is A Novel Translating Organelle Mediating Intercellular Communication. Y. Liu, R. Dahn[1], S. Park[1], E. Kurt[1], A. Presle[2], J. Gilbert[1], K. VanDenHeuvel[1], A. Jambhekar[3], J. Shivas[4], L. Qin[1], O. Olukoga[1], A. Echard[2], M. Blower[5], **A. R. Skop**[1]; [1]University Wisconsin-Madison, Madison, WI, [2]Instit Pasteur, Paris, FRANCE, [3]Harvard, Boston, WI, [4]Leica Microsystems, Buffalo Grove, IL, [5]Harvard, Boston, MA.

The midbody is a transient structure at the spindle midzone that is required for the terminal stage of cell division, cytokinesis. Long ignored as a vestigial remnant of cytokinesis, emerging data suggest midbodies are released from post-mitotic cells and can modulate cell fate decisions, proliferative state, tissue polarity, cilia formation, neuronal architecture and function, and oncogenesis. Our 'structure reveals function' approach first demonstrated that the midbody matrix- a mysterious electron-dense region of unknown composition- is the assembly site of a phase-separated biomolecular condensate, enriched for mRNAs encoding proteins involved in cytokinesis, oncogenesis and pluripotency regulation, including transcription factors. Hexanediol-sensitivity and FRAP analysis confirmed that the midbody matrix exhibits biophysical properties expected of an RNP condensate. SUnSET analysis of protein synthesis showed that both the mitotic and post-mitotic midbody are sites of active translation. Consistent with this, ribosomal subunits and elongation factors colocalize with puromycin labeling in a ring structure surrounding the midbody RNA core. We discovered that translation of midbody-enriched mRNAs is under tight temporal regulation, beginning in G1 after cells have formally exited mitosis, and continues after the midbody is abscised and released extracellularly from the daughters that birthed it. Our data suggest a model in which the midbody functions as a novel organelle with a complex life cycle comprised of both membraneless and membrane-bound phases: 1) an RNP condensate is assembled at the spindle overlap; 2) spatiotemporally regulated translation is initiated as the daughter cells leave mitosis and reenter G1; 3) abscission occurs and the membrane-bound RNP granule is released; 4) the midbody is bound, internalized, and transfers information to recipient cells.

Prokaryotic Cell Biology

P1679/B818

Z Ring Assembly Is Regulated by FtsZ Filament Binding Proteins.

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Cell division in bacteria is orchestrated by a group of proteins that work together to carry out cytokinesis and synthesize new cell wall at the division site. Filaments of FtsZ, a bacterial homolog of tubulin, form a "Z ring" at the middle of the cell that recruits other cell division proteins and constricts as the cell divides. While many of these proteins have been identified, the mechanisms of cell division remain poorly understood. Recent work has emphasized the importance of the properties of FtsZ filaments in the cytokinesis process. Using TIRF microscopy, we have shown that FtsZ filaments treadmill around the division site in the gram-positive bacterium *Bacillus subtilis*, and that these dynamics are of key

significance in bacterial cell division. Now, we investigate whether and how FtsZ filament assembly and treadmilling dynamics are regulated. We specifically investigate the FtsZ binding proteins, a group of proteins known to bind directly to FtsZ at the division site. These proteins have been proposed to regulate both FtsZ dynamics and bundling in *vitro*. To investigate this, we use live-cell single molecule lifetime measurements as a quantitative reporter of FtsZ's treadmilling dynamics independent of both spatial resolution and local filament density. We ask whether and how FtsZ binding proteins control filament structure and/or dynamics during the bacterial cell cycle. Surprisingly, our results suggest that these FtsZ binding proteins do not regulate FtsZ filament kinetics directly, but rather mediate Z ring assembly through filament bundling. Functional regulation of filaments by bundling, mediated by accessory filament-binding proteins, is a common feature of eukaryotic cytoskeletal systems, but has rarely been seen in bacteria. We propose that this regulated FtsZ filament bundling is a prerequisite for normal Z ring formation and cytokinesis.

P1680/B819

Single-molecule Imaging Reveals Distinct Subcomplexes of the *Bacillus Subtilis* Division Machinery.

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Despite decades of research, the fundamental molecular mechanisms underlying bacterial cytokinesis remain poorly understood. Recently, it has become clear that protein dynamics in the bacterial division complex, and in particular those of the tubulin homolog FtsZ, play a key role in this process. FtsZ polymers localize in a ring at future division sites and recruit cell-wall-synthesis enzymes that build a septum between daughter cells. FtsZ filaments move around this ring by treadmilling: subunits are added to one end of the filament and removed from the other. This results in the filament as a whole moving, but individual subunits remaining stationary. Filament treadmilling is required for the coincident motion of the associated cell wall synthesis enzyme Pbp2B around the division site, as well as efficient cell division. Strikingly, single molecules of Pbp2B move along with the treadmilling filament and are highly processive. Single-molecule processive motion along filaments is typically associated with molecular motors in eukaryotic systems, but neither Pbp2B nor any other protein associated with the division complex has homology to any known motors. How is this motion achieved? in addition to FtsZ, the division complex (or divisome) contains many other proteins whose dynamics have not been characterized. We imaged single molecule motions of each divisome protein using HaloTag fusions labelled with Janelia Fluor dyes. Two classes of motions were observed. Cytoplasmic proteins that bind directly to FtsZ remain immobile with FtsZ subunits. In contrast, the divisome proteins with periplasmic domains all move around the division site. This latter group contains the other division cell wall synthesis enzyme FtsW, and three non-enzymatic proteins which interact with one another but have unknown function. All four proteins move at the division site with velocities comparable to Pbp2B, and these directional motions require cell wall synthesis. We propose that these periplasmic proteins move around the cell in complex in a manner dependent on treadmilling FtsZ filaments. We are currently working to identify the molecular interfaces between the stationary and moving components. Additionally, we are uncovering how the localization and motion of these mobile proteins depends on specific FtsZ architectures.

P1681/B820

Cell Division Proteins Follow Treadmilling FtsZ Filaments by Diffusion-and-Capture.

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The tubulin homolog FtsZ plays a key role for bacterial cytokinesis, but the molecular mechanisms underlying its function are largely unknown. Treadmilling filaments of FtsZ are thought to actively move proteins within the division plane to distribute cell wall synthesis for the generation of the two new cell poles. To study how FtsZ filament dynamics are coupled to cell wall synthesis, we reconstituted part of the bacterial cell division machinery using its purified components FtsZ, FtsA and truncated transmembrane proteins essential for cell division. We found that the membrane-bound cytosolic peptides of FtsN and FtsQ co-migrated with treadmilling FtsZ-FtsA filaments. Remarkably, despite their directed behavior on the ensemble level, individual peptides showed random motion and transient confinement. Our work suggests that divisome proteins dynamically follow treadmilling FtsZ filaments at the cell division site by a diffusion-and-capture mechanism and highlights the importance of transient interactions for the self-organization of complex biological structures.

P1682/B821

Transpeptidase Pbp2 Governs Initial Localization and Activity of Major Cell-wall Synthesis Machinery in *Escherichia Coli*.

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Bacterial shape is physically determined by the peptidoglycan cell wall. The cell-wall-synthesis machinery responsible for rod shape in *Escherichia coli* is the processive 'Rod complex'. Previously, cytoplasmic MreB filaments were thought to govern formation and localization of Rod complexes based on local cell-envelope curvature. However, using single-particle tracking of the transpeptidase PBP2, we found strong evidence that PBP2 initiates new Rod complexes by binding to a substrate different from MreB or any known Rod-complex component. This substrate is likely the cell wall. Consistently, we found only weak correlations between MreB and envelope curvature in the cylindrical part of cells. Residual correlations do not require any curvature-based Rod-complex initiation but can be attributed to persistent rotational motion. Therefore, local cell-wall architecture likely provides the cue for PBP2 binding and subsequent Rod-complex initiation. We also found that PBP2 has a limiting role for Rod-complex activity, thus supporting its central role.

P1683/B822

LetB Forms a Tunnel for Lipid Transport Across the Bacterial Periplasm.

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Gram-negative bacteria are surrounded by an outer membrane composed of phospholipids and lipopolysaccharide (LPS), which acts as a barrier to the environment and contributes to antibiotic resistance. While mechanisms of LPS transport have been well characterized, systems that translocate phospholipids across the periplasm, such as MCE transport systems, are less well understood. Here we show that *E. coli* LetB (formerly YebT), uses multiple MCE domains to form a ~0.6 megadalton complex in the periplasm. Our cryo EM structure reveals that LetB consists of a stack of 7 modular rings, creating

a long hydrophobic tunnel through the center of the complex. LetB is sufficiently large to span the gap between the inner and outer membranes, and mutations that shorten the tunnel abolish function. Lipids bind inside the tunnel, suggesting that it functions as a pathway for lipid transport. Together, our results support a model in which LetB establishes a physical link between the bacterial inner and outer membranes, and creates a hydrophobic pathway for the translocation of lipids across the periplasm, supporting the integrity of the outer membrane permeability barrier.

P1684/B823

Liquid-Like Protein Behavior Involved in the Spatial Regulation of Carbon-fixing Organelles.

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Carbon fixation is one of the most fundamental biological processes, and is almost entirely catalyzed by the enzyme RuBisCO. While typically associated with plants, nearly half of all carbon fixation is carried out by bacteria. However, bacteria fix carbon via a slightly different mechanism, as they have evolved specialized organelles known as carboxysomes to aid in the process. These carboxysomes function by concentrating and encapsulating RuBisCO and CO2 within a protein shell, effectively saturating RuBisCO with its substrate to create a highly efficient carbon-fixing reaction compared to plants. As a result, carboxysomes have piqued interest for engineering alternative and more efficient carbon-fixing pathways to address the climate crisis and need for green energy. Despite their applications and importance in the global carbon cycle though, little is known about how carboxysomes form or are spatially regulated in carbon-fixing bacteria. A recent focus of ours has been the spatial regulation of carboxysomes in the model cyanobacterium *Synechococcus elongatus.* We have identified the maintenance of carboxysome distribution system (McdAB) as the two-factor system that equally spaces carboxysomes down the cell to ensure their inheritance after cell division. The first factor, McdA, is an ATPase that coats the nucleoid. The second factor, McdB, binds carboxysomes and removes McdA from the nucleoid in the vicinity of each carboxysome. The resulting McdA gradients drive the positioning of carboxysomes. Although we have identified the two-factor system, the specific interactions behind gradient formation and carboxysome positioning remain unknown. My results indicate that McdB, the protein that associates with carboxysomes, undergoes liquid-liquid phase separation (LLPS), a physical process by which proteins condense into their own liquid phase. The results I cover here show McdB displaying hallmark liquid behaviors (e.g. droplet fusions, wetting, internal diffusions, etc.) that are highly responsive to physical conditions such as pH and salt levels. Interestingly, carboxysomes have also been shown to undergo LLPS to facilitate their assembly. These data are striking since our results indicate that McdB interacts strongly with the components responsible for driving carboxysome LLPS. It is further known that carboxysomes facilitate and respond to changes in cytoplasmic pH. Taken together, these data lead us to hypothesize that local fluctuations in pH may regulate LLPS in relation to McdB and carboxysome assembly and spatial regulation.

P1685/B824

Protein Gradients on the Nucleoid Position the Carbon-fixing Organelles of Cyanobacteria.

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Carboxysomes are protein-based bacterial organelles encapsulating key enzymes of the Calvin-Benson-Bassham cycle. Previous work has implicated a ParA-like protein (hereafter McdA) as important for

spatially organizing carboxysomes along the longitudinal axis of the model rod-shaped cyanobacterium *Synechococcus elongatus* PCC 7942. Yet, how self-organization of McdA emerges and contributes to carboxysome positioning is unknown. Here, we identify a small protein, termed McdB that localizes to carboxysomes and drives emergent oscillatory patterning of McdA on the nucleoid. Our results demonstrate that McdB directly stimulates McdA ATPase activity and its release from DNA, driving carboxysome-dependent depletion of McdA locally on the nucleoid and promoting directed motion of carboxysomes towards increased concentrations of McdA. We propose that McdA and McdB are a previously unknown class of self-organizing proteins that utilize a Brownian-ratchet mechanism to position carboxysomes in cyanobacteria, rather than a cytoskeletal system. We further demonstrate that McdA/B levels influence carboxysome ultrastructure and size. Finally, we show that the McdAB system is widespread among all known cyanobacterial taxonomic orders and morphologies and that McdB is an intrinsically disordered protein across evolutionary time. These results have broader implications for understanding spatial organization/biogenesis of protein mega-complexes and organelles in bacteria.

P1686/B825

Xylan Metabolism by *Caulobacter Crescentus*.

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Bacteria present unique opportunities to explore the molecular mechanisms of nutrient acquisition processes at single cell level under various experimental conditions. *Caulobacter crescentus* is a gram-negative oligotrophic environmental bacterium with adaptive physiological responses, like slow growth or complete arrest of cell cycle; the presence of sessile and motile progenies, with the sessile cells growing stalk to enhance nutrient uptake for growth in low-nutrient conditions. Details of carbon source, such as cellobiose, maltose, and xylose, utilization by *C. crescentus* can be found but molecular details or mechanisms of polysaccharide, such as xylan, utilization has not been reported. Database analyses indicate *C. crescentus* has a repertoire of xylanases and carbohydrate-modifying enzymes, along with membrane-bound transporters for efficient breakdown and uptake of xylan. Growth and metabolic properties of *C. crescentus* in M2 minimal medium containing xylan as the sole carbon source was investigated. Growth curves of *C. crescentus* in xylan show typical phases as seen in other mono- and disaccharide carbon sources in similar conditions but with significantly extended lag phase. Enzyme assays using *p*-nitrophenol xylopyranoside substrate and xylo-oligosaccharides show high level expression of periplasmic xylosidases on the second day of growth on xylan substrate coinciding with the beginning of the log phase as well as possible membrane-bound xyloside hydrolases as well as glucuronidase. Gene expression analysis of cells grown in xylan show expression of a select number of xylanases, suggesting induced expression of genes. 2D gel electrophoresis based proteomic and genomic database analyses further revealed several xylosidases as well as TonB-dependent receptor mediated transporters expression indicating that these enzymes and proteins work in a concerted manner to access metabolizable xylose and other monosaccharide derivatives. Chromatography (HPLC & TLC) and ^{1}H-NMR based metabolomic analyses of the extracellular medium combined with enzyme assay data suggest xylan binding onto the bacterial surface and xylan fragment uptake across the outer membrane. A comprehensive model emerges for *C. crescentus* that resembles both gut and environmental bacterial xylan utilization models further advancing the molecular level understanding of xylan derived nutrient acquisition in bacteria.

P1687/B826

A Molecular Rack and Pinion Machinery Enables Bacterial Gliding Motility.

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Bacterial gliding has fascinated biologists and physicists for a long time, yet we know very little about its mechanism. The gliding bacterium *Flavobacterium johnsoniae* is known to have an adhesin, SprB, that moves along the cell surface on a spiral track. When cells are sheared, they stop gliding but can be tethered by addition of an anti-SprB antibody. Tethered cells spin about 3 Hz. We labeled the Type 9 protein secretion system (T9SS) with a yellow-fluorescent-protein (YFP). When labeled cells were tethered, a YFP spot was found near the rotation axis, which shows that the molecular motor that drives rotation localizes with the T9SS. The spiral track was labeled by following the motion of Cy3-labeled SprB. The distance between the rotation axis and the track was determined by a measurement involving both labels, YFP and Cy3, yielding 90 nm. If a rotary motor spins a pinion of radius 90 nm 3 Hz, a spot on its periphery will move 1.5 μm/s, the speed at which cells glide. Our data argue for the presence of a molecular rack and pinion machinery. The pinion drives a flexible tread that carries SprB along a track fixed to the cell surface. Cells glide when such an adhesin adheres to the solid substratum.

P1688/B827

Structural, Functional and Transcriptional Characterization of Novel Identified hipBAXn2 Toxin-Antitoxin (TA) Module from *Xenorhabdus nematophila*.

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This is the first report to characterize genomic *hipBA*^{Xn2} TA module from *Xenorhabdus nematophila*, a species-specific mutualist of entomopathogenic nematode *Steinernema carpocapsae*. *hipBA*^{Xn2} was identified in the genome of *X. nematophila* ATCC 19061 (NCBI Refseq NC_014228) at position 3774379-3775635 bp. It is a type II TA module which consists of *hipA*^{Xn2} toxin gene encoding 270 amino acid residues protein and *hipB*^{Xn2} encoding antitoxin of 135 amino acid residues protein. Structural models for both proteins were deduced by threading approach and molecular movements of models were also validated by Molecular Dynamics (MD) simulation analysis. For functional analysis *hipA*^{Xn2} toxin, *hipB*^{Xn2} antitoxin and an operon having both genes were cloned in pBAD/His (c) vector and transformed in *Escherichia coli* TOP10 cells. All proteins were purified by using Ni-NTA affinity chromatographic technique and confirmed by Western blot analysis using anti-His antibody. Purification results showed the yield of of 0.37 mg/mL and 0.535 mg/mL for recombinant HipA^{Xn2} toxin and HipB^{Xn2} antitoxin protein respectively. For endogenous toxicity assay of *hipBA*^{Xn2} TA module, growth profile and cell viability of *E. coli* cells expressing recombinant HipA^{Xn2} toxin and HipB^{Xn2} antitoxin proteins was studied. After 4h of induction with 0.2% L-arabinose, cells expressing HipA^{Xn2} toxin retard the growth (measured as CFU and OD_{600}) by more than 2.5-fold as compared to the control cells and as induction time period increased, difference between cell viability was also increased. However, cells expressing both *hipA*^{Xn2} toxin and *hipB*^{Xn2} antitoxin genes simultaneously, HipB^{Xn2} antitoxin protein reduced the lethal toxic effect of HipA^{Xn2} toxin by more than two- fold. To determine the active amino-acids residues responsible for HipA^{Xn2} toxicity, site-directed mutagenesis (SDM) was performed. It was observed that amino-acid H164, N167 and S169 were key residues for the toxicity of HipA^{Xn2} toxin. In transcriptional analysis, we have predicted 529 bp upstream region of *hipBA*^{Xn2} and *LacZ* reporter construct was prepared. *LacZ* assay was

performed in different stress conditions like elevated temperature, nutrient deprivation and antibiotic treatments. In all the three stress conditions, promoter of *hipBA*^{Xn2} TA module was found significantly up-regulated as compared to normal growth condition. Auto regulation mechanism of *hipBA*^{Xn2} module was also confirmed by *in-vitro* Electrophoretic Mobility Shift Assay (EMSA), where 529 bp promoter region was interacting with purified recombinant HipA^{Xn2} toxin and HipB^{Xn2} antitoxin proteins. This study provides a comprehensive characterization of *hipBA*^{Xn2} TA module comprising a bright spotlight towards structural, functional and transcriptional features.

P1689/B828

***In Silico* Identification of Novel Cry Toxins and Assessment of Domain-specific Evolutionary Impact on the *Bacillus Thuringiensis* Toxin Adaptive Radiation.**

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Cry toxins pose major proteinaceous virulence factors of the insecticide bacterium *Bacillus thuringiensis*, a potent biopesticide widely used for crop protection. These proteins affect numerous species among Insecta class including those of orders Leipdoptera, Diptera, Hymenoptera and Coleoptera as well as several mite species and nematodes of Rhabtida order. To date, several prominent groups of Cry toxins have been identified of which three domain toxins (3D-Cry) prevail with more than 700 entities already discovered. Just like their name supposes, 3D-Cry toxins consist of three domains, with domain I responsible for toxin oligomerization and pore formation and domains II and III involved in binding to insect midgut receptors. Despite Cry toxins being excessively studied, neither their evolutionary origin and diversification nor mechanisms underlying their host specificity have been elucidated plausibly so far. Beyond that, due to their structural similarity Cry toxins tend to be misannotated in genomic and proteomic data indicating that a vast part of their true diversity is being overlooked. In the present work, we stepped towards expansion of existing Cry toxin database and revealing their evolutionary relationship. Recently, we have developed an HMM-based tool capable of mining novel toxins from both assembled sequences and raw reads. By applying this tool to all open access *Bacillus*-related sequences we managed to find more than 300 undescribed entities. The same tool was then used for performance of domain layout of both novel and trivial toxins to obtain sequences standing for mature processed toxins. Maximum likelihood phylogenetic trees of full-size processed toxins underpinned the discovery of novel high-rank groups of Cry toxins, while domain-restricted trees indicated unequal identity rate distribution and dN/dS ratio in three Cry domains implying differences in their contribution to protein radiation and putative host specificity, with domain III surprisingly being more conservative among neighboring clades than two other domains. Taken together, these data might shed light on all three Cry toxin actual diversity, evolutionary radiation and host specificity development. This work was supported by the Russian Science Foundation (Grant No 18-76-00028).

P1690/B829

Insights into the Zinc Homeostasis of *Bacillus an thracis by* Characterization of Its Zinc Uptake Regulator.

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Zinc (Zn) has abounding occurrence in the living systems. It plays paramount roles including catalytic, structural and regulatory, both in prokaryotes and eukaryotes. Many Zn-dependent proteins are known to manifest virulence in pathogenic bacteria. In addition to the varying Zn levels in the external environment, the pathogenic bacteria encounters Zn inconstancies within the host i.e. either hyper- or hypo-zincemic conditions. Thus for successful survival, maintenance of Zn homeostasis is imperative at all times. Zn uptake regulator (Zur), a Fur family transcriptional regulator, is connoted in maintaining Zn homeostasis in pathogenic bacteria. Zn homeostasis has been marginally scrutinized in *Bacillus anthracis*, the top-rated bio-terror agent and causative organism of the fatal disease anthrax, with no decipherment of the role of Zur. BAS4181 is annotated as Zn-specific transcriptional regulator, further substantiated by computational and experimental analyses. BAS4181 gene (*ba zur*) lies *in* a three-gene operon, co-transcribed with *znuB* and *znuC* homologs, the components of a high-affinity Zn ABC transporter. The residues critical for Zn and DNA binding were delineated by homology modelling and sequence/structure analysis. Purified BaZur prodigiously exists in dimeric form as indicated by size exclusion chromatography and blue native-polyacrylamide gel electrophoresis. Computational and manual strategies were employed to decipher the putative regulon of *ba zur*, comprising of 11 genes, controlled by 6 promoters, each harbouring at least 1 Zur box. The DNA binding ability of BaZur to the promoter regions of the regulon candidates and itself was ascertained by electrophoretic mobility shift assays. Most of the regulon genes lacked functional annotation hence comprehensive in *silico* analysis was employed, revealing their role in Zn uptake, mobilization and as Zn chaperones. BaZur was found to exert negative regulation on most of the regulon genes including it. A downregulation was observed in the expression of *ba zur* under Zn excess conditions and marked upregulation under N, N, N′, N′-Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) mediated Zn-depleted environment, adding credence to its negative autoregulation. Moreover, an increase in the transcript levels of the regulon genes upon exposure of cells to TPEN connoted their role in combating hypo-zincemic conditions. Therefore it is proposed that under conditions of Zn feast, Zn bound BaZur represses the expression of its regulon genes and under Zn famine, BaZur exists in non-Zn bound form, causing their derepression. Thus, this study provides an insightful investigation of Zur and its connotation in Zn homeostasis in *B. An thracis*.

P1691/B830

Identification and Characterization of Marine Bacteria Associated with Sea Urchin *Lytechinus variegatus*.

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Many symbiotic bacteria coexist on the surfaces of sessile marine invertebrates and are rich sources of unusual metabolites. This study focuses on identifying and characterizing bacterial flora that coexist on the tests and orifices of the sea urchin *Lytechinus variegatus*. Several bacterial strains were isolated from wild type sea urchins by gently swabbing their external surfaces and plating them onto marine agar plates. Out of the initial samples, 14 were chosen for identification and characterization. All 14 bacteria were gram negative rods. They tested positive for the oxidase tests and did not ferment sucrose. None of the tested bacteria showed any antibacterial properties. Kirby-Bauer tests revealed that many were resistant to both common and some rare antibiotics. Some of the isolated bacteria harbored endogenous cryptic plasmids of around 20 Kbp. When grown on starch and cellulose plates, some exhibited amylase and cellulase activities. One of the isolated bacteria was agarolytic in nature. The conserved 500bp of the 16S rDNA was sequenced for all 14 bacteria samples. Future studies involve

phylogenetically identifying these bacteria isolates. The raw DNA sequence obtained will be analyzed against an up-to-date bioinformatics database using an open source software, namely QIIME2. This includes taxonomic assignment, phylogenetic reconstruction and diversity analysis and visualizations. Additional tests might include antimicrobial assays to further determine its sensitivities to other microbial agents.

P1692/B831

***E. coli* Mutant Library Screen with Lacritin Bactericidal Peptide N-104 Identifies Potential Roles for PotH and Common Virulence Factor FeoB in N-104 Dependent Cell Death.**

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Antimicrobial peptides play important roles in the innate defense of multicellular organisms. Recently, we discovered that a cleavage potentiated fragment of the prosecretory mitogen lacritin in tears, known as 'N-104', is bactericidal towards *E. coli*, *P. Aeruginosa* and *S. epidermidis* (J. Biol. Chem. 289:22172-22182, 2014), thereby substantially contributing to the sterility of human tears. To elucidate the mechanism-of-action, we screened for N-104 resistant mutants among 3,985 single gene knockout mutants of the *E. coli* K-12 Keio collection. Although effective at lower doses, 100 µM N-104 was applied to ensure complete killing of ~10^6 cfu/ml suspensions. AlamarBlue served as the end-point assay to monitor bacterial cell viability and proliferation. Fluorescence of a 200 µl suspension of each knockout strain was monitored in duplicate for 5 hours at 34°C (surface temperature of the eye) after which N-104 was added and viability monitored for the next 5 hours. A viability slope ratio of >0.75 (treated to untreated) distinguished several resistant mutants including *potH* and *feoB*, and uncharacterized *yhfZ*, *ybdM* and *ybaE*. FeoB is ferrous iron transport protein B, a virulence factor for *P. Aeruginosa* in chronic cystic fibrosis and of *E. coli* in urinary tract infections. The PotH is a membrane protein subunit for transporting putrescine which is strongly involved in proliferation and viability of bacteria. How N-104 dependent cell death is initiated by FeoB or PotH at the inner membrane of *E. coli* for presumed transduction to other gene products or via metabolites remains to be determined.

P1693/B832

Characterization of Antibiotic Resistant Bacteria Present on the Lane College Campus.

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Antibiotics are medications that destroy or slow down the growth of bacteria. Beta-lactam antibiotics, such as penicillin, consist of a beta-lactam ring that inhibits transpeptidase, an enzyme needed for cell wall biosynthesis in bacterial organisms. Since antibiotics are among the most highly prescribed medication, many pathogenic bacteria have developed resistance towards them. The soil is a natural reservoir of antibiotic resistant bacteria, and characterization of these bacteria can enhance our understanding of the evolution of antibiotic resistance. Our goal for this project is to isolate and characterize soil bacteria that are resistant to beta-lactams. We hypothesize that antibiotic resistant bacteria are present in the soil in the Lane College campus. Furthermore, we hypothesize that these bacteria have a beta-lactamase gene, which is an enzyme that inactivates the bacteria by cleaving the beta-lactam ring. We collected soil samples from the Lane College campus and cultured the bacteria a nutrient agar at room temperature. To screen for antibiotic resistant bacteria in this culture, we then replica plated the bacteria onto nutrient agar containing 100 ug/ml of ampicillin. We then selected two

colonies for further characterization. We tested the bacteria for resistance to additional beta-lactams, penicillin and amoxicillin. We then further characterized by Gram staining and morphological observation. Lastly, we used PCR to screen for the presence of beta-lactamase genes, TEM-1 and Bla-1, two common beta-lactamases. The two strains were resistant to ampicillin, penicillin, and amoxicillin. Both samples were Gram negative and the cells were a coccus shape. The beta-lactamase genes TEM-1 and Bla-1 were not detected by PCR. We are planning to use the 16S rDNA gene to identify the bacteria, and to look for presence of other beta-lactamase genes and further characterize the transpeptidase gene. This work is supported by NSF HRD 1623340 to MVS.

P1694/B833

Effect of *Xylopia Aethiopica* Aqueous Fruit Extract on Bacterial Growth Kinetics.

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Xylopia aethiopica is an evergreen, aromatic tree of the an nonaceae family. It is native to the lowland rainforest and moist fringe forests in the savanna zones of Africa where it is widely eaten and used in traditional medicine. The essential oil of *X. Aethiopica,* extracted from the seeds or the bark, has been shown to have antiseptic activity. The purpose of this project was to measure the effect of an aqueous *Xylopia* fruit extract on growth kinetics of gram-negative *Escherichia coli* and gram-positive *Bacillus subtilis. The* extract was prepared by grinding the *Xylopia* fruit and boiling in distilled water for 30 minutes. Liquid cultures of *E. coli* and *B. subtilis* were each grown in microplate wells at 37 °C with shaking in either the presence or absence of the *Xylopia* extract. Bacterial growth was monitored over the course of seven hours by measuring optical density at 600 nm using a BioTek microplate reader. Results demonstrated that whereas growth of *E. coli* was slightly suppressed in the presence of the extract, growth of *B. subtilis* was completely inhibited. Experiments were repeated several times yielding the same results. It was concluded that the aqueous *Xylopia* fruit extract exerted differential antibacterial activity on these strains, possibly due to their differences in cell wall structure. Further experiments will examine growth kinetics of different gram-positive and gram-negative strains to address this question.

P1695/B834

Tick-borne Lyme Disease: Investigating the Role of Micronutrients On*Borrelia Burgdorferi* Population Growth.

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Lyme disease (LD), caused by the spirochete *Borrelia burgdorferi* (*B.b.*) that is transmitted by *Ixodes* species ticks, is the most common vector-borne illness in the U.S., Europe, and in the Northern Hemisphere. Recently revised estimates from the CDC suggest that there are likely over 300,000 new cases per year in the U.S., but the actual number is probably much higher as many cases are underreported and undiagnosed. Clinical data indicates that certain vitamins affect the outcome of antibiotic treatment of LD, while other clinical work recounts that levels of serum vitamins for LD patients differ from healthy patients. As LD is so prevalent, especially in Appalachia, with not many researchers studying it, it is important to investigate Tick Borne LD *B.b. In*teraction and effects with vitamins, which could lead to a better approach to treatment and effectiveness of antibiotics. The goal of this study is to examine the direct effects of vitamins on *B.b*. populations when cultured with micronutrients. I hypothesized that the vitamins A, B, B6, B12, C and E would have a positive effect upon

*B.b.*growth, as shown by an increase in the *B.b.* count. I also expected the results to confirm that vitamin D3 and Ca would have a negative effect upon the *B.b.* population counts, therefore a decrease in counts. It is important to note in the final results that positive *B.b.* growth, therefore an increase in counts correlate to a negative outcome for LD patients, and a decrease in *B.b.* population counts are a positive outcome for the patient with decreased bacteria in the patient. The results showed that the B vitamins & Ca have a positive effect on the *B.b.* counts, thus, would cause the *B.b. To* multiply and worsen the disease in the patient. Also, vitamins A, C, E, and D3 had a negative effect on the *B.b.* counts, and therefore a patient with LD could benefit by taking these vitamins to combat the *B.b.* growth. The implications of this project are key, as the CDC recently released a study that LD-carrying ticks are now in half of all U.S. counties. If patients with LD can take a vitamin that can decrease *B.b.* counts, then this can help patients not only in Appalachia, but those without access to higher level medical care including proper antibiotics. This has not previously been studied individually and research on this widespread, harmful, understudied disease gives valuable insight into a disease affecting many especially the local population of Appalachia.

P1696/B835

Flagellum Is Responsible for Promoting Effects of Viable *Escherichia Coli* on Calcium Oxalate Crystallization, Crystal Growth and Aggregation: an in *Vitro* Model for Kidney Stone Formation.

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Urease-producing bacteria (especially *Proteus mirabilis*) can cause infection kidney stone. However, recent studies have shown that intact viable non-urease-producing bacteria such as *Escherichia coli* could also promote calcium oxalate (CaOx) kidney stone formation but with unclear mechanism. We thus hypothesized that some relevant bacterial components might be responsible for such promoting effects of the intact viable *E. coli*. Flagellum, capsule, lipopolysaccharide (LPS), and outer membrane vesicles (OMVs) were isolated/purified and their stone modulatory activities were evaluated using CaOx crystallization, crystal growth, and crystal aggregation assays. Among these, flagellum had the most potent promoting effects on CaOx crystallization, crystal growth, and crystal aggregation. Validation was performed by deflagellation demonstrating that the deflagellated intact viable *E. coli* had markedly reduced CaOx crystal modulating activities in all aspects, comparable to those of the negative controls. These findings provide the direct in *vitro* evidence indicating that flagellum is responsible for promoting effects of viable *E. coli* on CaOx crystallization, crystal growth and aggregation, which are the important mechanisms for kidney stone formation.

P1697/B836

Far-Red Light Photoacclimation in Endolithic Cyanobacteria.

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In hyper-arid deserts, cyanobacteria colonize the pore spaces of rocks to exploit their water retention properties and shield themselves from harsh winds and solar radiation. These endolithic cyanobacteria provide carbon to the associated microbial community and photosynthesize using chlorophyll a, phycobilisomes, and two major photosystems (PS I & PS II). We previously found that the solar spectrum within the colonization zone of rocks is shifted towards far-red light (>710nm), which chlorophyll a is

unable to absorb. To understand how this light condition influences photosynthesis, we analyzed enrichment cultures grown under white and far-red light with spectrophotometry, genome sequencing, RT-qPCR, and pigment extractions. Our analysis revealed the presence of chlorophyll f and a canonical 21-gene cluster that allows these endolithic cyanobacteria to make use of light in the far red. We also found that chlorophyll f synthase, which catalyzes the conversion of chlorophyll a into f, was highly expressed only in cells exposed to far red light, demonstrating the ability of endolithic cyanobacteria to use far-red light to photosynthesize.

P1698/B837

Investigation of Potential for Green Tea and Honey as Natural Anti-biofilm Agents.

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Biofilm is an accumulation of multicellular and sometimes multi-species microbial mass embedded in polysaccharide matrix with adhesive abilities; this matrix is known as “extracellular polymeric substance” (EPS). Complex biofilm has remarkable ability to impede normal immune responses and aggressive anti-microbial medical interventions, leading to difficulties in treating wound infections. Honey and green tea extract have been previously identified as natural substances that appear to possess anti-biofilm properties with potential for promotion of wound healing. Previous studies have targeted the ability of green tea and/or honey to prevent single-species biofilm formation, which could be purely an antibiotic effect rather than targeting EPS production. In wound infections, biofilm typically involves multiple species of microorganisms. Here we utilize in *vitro* biofilm growth and mixed bacterial cultures to gain further understanding of the potential for prevention and/or eradication of biofilm using green tea and honey. The model strains of bacteria used in our study are: *Staphylococcus epidermidis* RP62A (WT)--a robust biofilm former, *Staphylococcus epidermidis* (ATCC 12228)--a very weak biofilm former, *Staphylococcus aureus* (ATCC 6538)--a moderate biofilm former and known wound pathogen, and *Pseudomonas aeruginosa* (ATCC 9027)--a moderate biofilm former and known wound pathogen. With all species incubated under the same testing conditions, our preliminary findings suggest that 20% green tea extract is capable of preventing single-species bacterial growth and biofilm formation when applied to planktonic cultures, yet does not appear to be capable of disrupting established biofilms. In contrast, 1% honey has ability to prevent biofilm formation in all three Staphylococcal species, and is capable of disrupting established Staphylococcal biofilm. In mixed-species cultures, co-incubation of *S. Aureus* with *S. epidermidis* RP62A appears to have an antagonistic effect on the ability of RP62A to form a robust biofilm. In contrast, co-incubation of *P. Aeruginosa* with *S. Aureus* appears to have a synergistic effect, allowing formation of a more robust biofilm than *S. Aureus* alone. With all mixed cultures, 20% green tea extract and 3% honey are both capable of preventing biofilm formation when added to planktonic mixed cultures before incubation, 3% honey reduces biomass of established mixed biofilm, but 20% green tea has essentially no effect on established mixed biofilm. Future studies will investigate the effects of green tea and honey on multi-species biofilm that naturally forms in infected "wounds" using *ex vivo* zebrafish explants cultures.

P1699/B838

Characterization of Amino Acids Involved Polymer Nucleation and Turnover for Tubz, a Bacterial Tubulin Homolog That Powers Plasmid Partitioning.

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TubZ is a tubulin homolog that moves plasmids, phage genomes or virions inside of bacterial cells. In *Bacillus thuringiensis*, TubZ polymers treadmill and help partition a large virulence plasmid. In a system reconstituted in vitro, DNA from these plasmids can associate with one end of a TubZ filament and be pulled along as the filament grows at the other end. TubZ initially assembles into GTP-containing, two-stranded filaments. After GTP hydrolysis, the polymers rearrange to form a four-stranded filament before finally disassembling. TubZ has an extended C-terminal tail that forms connections between subunits along a protofilament. This tail appears to play a role in filament assembly, rearrangement and disassembly. We are mutating amino acids predicted to be necessary for each stage in assembly and then characterizing the effects on subunit assembly and GTP turnover. Results to date support the idea that GTP turnover is limited by the stability of the 4-stranded filaments. Mutations that destabilize the 4-stranded filaments can increase GTP turnover up to 40-fold. The extended C-terminal tail may also affect the stability of 4-stranded filaments, in addition to affecting the nucleation of hydrolysis-competent filaments. By deleting a portion of this tail or by mutating amino acids that might interact with the tail, the GTP turnover rate can be raised 5-fold, or the critical concentration for GTP hydrolysis can be either raised or lowered 5-fold without affecting steady state GTP turnover rates. We are continuing to define how of these and other mutations affect TubZ assembly and disassembly.

P1700/B839

Degradation of Cyanogens by Bacteria Isolated from the Solid Waste Leachates of a Landfill Site in Lagos, Nigeria and an alysis of the Unculturable Microbial Communities.

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Landfills are environmental repository for most organic and inorganic discarded materials. Microorganisms are responsible for the degradation of organic compounds in landfills. Despite the known toxicity of cyanide to man, it is still used and released into the environment by mining, galvanic and chemical industries. Bacterial strains capable of utilizing glutaronitrile as the sole source of carbon and nitrogen were isolated from solid waste leachates (SWL) by selective enrichment culture. Isolates were grown in media containing glutaronitrile and benzonitrile. Samples were processed for metagenomic DNA extraction which were followed by 16S rDNA amplification and sequencing. *Bacillus* sp. WOD8 KX774193 and *Corynebacterium* sp. WOIS2 KX774194 strains were obtained and both demonstrated broad specificities for nitriles degradation. Nitralase genes in WOD8 and WOIS2 were amplified as 1400bp and 1000bp respectively and then sequenced. The result of metagenomic analysis showed that twenty megabyte (20Mb) of data (2x300bp long paired end reads) was produced from the pooled sample with bacteria constituting 99.87% of the total read counts (i.e. 74766 read count). These findings provide genetic evidence of the involvement of WOD8 and WOIS2 in the degradation of the cyanogens. The study also revealed a high diversity of bacterial community in a major landfill in Lagos with potential that can be harnessed for cyanide containing waste management strategies.

04

Host-Pathogen / Host-Commensal Interactions 1

P1701/B840

Single Cell Lineage Dynamics of the Endosymbiotic Cell Type in a Soft Coral *Xenia* Species.

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Many hard and soft corals harbor algae for photosynthesis. The algae live inside coral cells in a specialized membrane compartment called symbiosome, which shares the photosynthetically fixed carbon with coral host cells, while host cells provide inorganic carbon for photosynthesis. This endosymbiotic relationship is critical for corals, but increased environmental stresses are causing corals to expel their endosymbiotic algae, i.e. coral bleaching, leading to coral death and degradation of marine ecosystem. To date, the molecular pathways that orchestrate algal recognition, uptake, and maintenance in coral cells remain poorly understood. We report chromosome-level genome assembly of a fast-growing soft coral, *Xenia* species (*sp.*), and its use as a model to decipher the coral-algae endosymbiosis. Single cell RNA-sequencing (scRNA-seq) identified 13 cell types, including gastrodermis and cnidocytes, in *Xenia sp*.Importantly, we identified the endosymbiotic cell type that expresses a unique set of genes implicated in the recognition, phagocytosis/endocytosis, maintenance of algae, and host coral cell immune modulation. By applying scRNA-seq to investigate algal uptake in our new *Xenia sp.* regeneration model, we uncovered a dynamic lineage progression from endosymbiotic progenitor state to mature endosymbiotic and post-endosymbiotic cell states. The evolutionarily conserved genes associated with the endosymbiotic process reported herein open the door to decipher common principles by which different corals uptake and expel their endosymbionts. Our study demonstrates the potential of single cell analyses to examine the similarities and differences of the endosymbiotic lifestyle among different coral species.

P1702/B841

Putative Hookworm (*Ancylostoma Ceylanicum*) G-protein-coupled Receptor Expression in *Caenorhabditis Elegans*.

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Many parasites, including hookworms of the genus an *cylostoma*, are specialists that require host-specific signals to resume development upon infection. Although little is known about the nature of these signaling mechanisms, evolutionary conservation between hookworms and the model nematode, *Caenorhabditis elegans,* suggests that hookworm host-signal receptors are likely to be G-protein coupled receptors (GPCRs) expressed in amphid neurons. Based on analysis of developmental stage-specific RNA-Seq data from *A. caninum* and *A. ceylanicum*, predicted hookworm GPCRs with increased expression levels during the infective larval stage 3 (iL3) were identified. Stable transgenic lines of *C. elegans* transformed with putative promoter-GFP transcriptional fusions of candidate *A. ceylanicum* GPCRs were established. Of nine hookworm promoter fusions tested, four conferred differential GFP expression in *C. elegans* tissues, including the digestive tract, pharynx, and neuron pairs during both normal development and dauer arrest. The putative GPCR encoded by one neuronally expressed gene is

upregulated in both *A. ceylanicum* and *A. caninum*, suggesting that it may play a general role in hookworm development during host infection. To confirm protein localization, a carboxy terminal GFP-fusion of a neuronally-expressed, putative hookworm GPCR was generated using the 5' intergenic sequence and the entire coding sequence, including introns. Within the five independently transformed transgenic lines examined, no GFP expression was observed during normal growth or dauer developmental arrest. An amino terminal GFP translational fusion with a Gly-Ser linker has been generated to reassess protein expression. This work demonstrates that hookworm promoters can differentially regulate gene expression in *C. elegans*, providing a heterologous system for the study of hookworm gene expression patterns.

P1703/B842

***Caenorhabditis Elegans* Innate Immune Response to *Bartonella Bacilliformis*.**

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The genus *Bartonella* encompasses aerobic or microaerophilic, Gram-negative, facultative intracellular bacteria with a peculiar intraerythrocytic lifestyle. These pathogens have been detected in a wide range of animals. *Bartonella bacilliformis* (*Bb*) is among the species that can affect humans. Carrion's disease, a two-stage disease that encompasses the Oroya fever and Peruvian warts, is caused by *Bb*. This pathology can result in death for more than 80% of infected patients if they are not treated with antibiotics; likewise, its increase in the pediatric population is alarming. *Bb* is known to evade the innate immune system of its host. A primary amino acid sequence change in the flagellin prevents the flagella from being recognized by the Toll-like receptor (TLR) thus promoting its evasion. After it bypasses the innate immune system, *Bb* is said to find its niche in the endothelial cells. During *Bb* infection, signs of endothelial cell proliferation are evidenced by vascular tumors known as Peruvian warts. In *vitro* models have shown that *Bb* stimulates endothelial cell growth and also induces angiogenesis. In this research, *Caenorhabditis elegans* (*C. elegans*) is used to study the host-pathogen factors determining susceptibility to *Bb* infections. In our study, we exposed wild-type *C. elegans* to both dead and alive *Bb* and non-pathogenic *E. coli OP50*, simultaneously. We determined that *C. elegans* preferred alive *E. coli* instead of alive *Bb*. Over 48 hours, 81.5% of the *C. elegans* chose the alive *E. coli* as opposed to a 9.2 % that chose the alive *Bb*. No nematodes were found on the dead *E. coli* or *Bb*. The remaining *C. elegans* were found outside of either bacteria. These findings suggest that nematodes have an aversion towards *Bb*. Experiments are being conducted to determine the innate immunity pathways used by *C. elegans* (p38 MAPK, DAF-2/DAF-16, TOL-1) in response to *Bb* infections.

P1704/B843

***Leishmania Amazonensis* Hijacks Host Cell Lysosomes Involved in Plasma Membrane Repair to Induce Invasion in Fibroblasts.**

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Intracellular parasites of the genus Leishmania are the causative agents of human leishmaniasis, a widespread emergent tropical disease. The parasite is transmitted by the bite of a hematophagous sandfly vector that inoculates motile flagellated promastigote forms into the dermis of the mammalian host. After inoculation, parasites are ultimately captured by macrophages and multiply as round-shaped

amastigote forms. Macrophages seem not to be the first infected cells since parasites were observed invading neutrophils first whose leishmania-containing apoptotic bodies were latter captured by macrophages, thereby becoming infected. The fact that *Leishmania* spp are able to live and replicate inside immune phagocytic cells and that macrophages are the main cell type found infected in chronicity created the perception that Leishmania spp are passive players waiting to be captured by phagocytes. However, several groups have described the infection of non-phagocytic cells in vivo and in vitro. The objective of this work was to study the cellular mechanisms involved in the invasion of non-professional phagocytes by *Leishmania*. We show that promastigotes of *L.amazonensis* actively induces invasion in fibroblasts without cytoskeleton activity, thus by a mechanism that is distinct from phagocytosis. Inside fibroblasts parasites transformed in amastigotes, remained viable for at least two weeks and re-transformed in promastigotes when returned to insect vector conditions. Similarly to what was observed for *T. cruzi,* infection involves calcium signaling, recruitment and exocytosis of lysosomes involved in plasma membrane repair and lysosome-triggered endocytosis. Conditions that alter lysosomal function such as cytochalasin-D and brefeldin-A treatment or the knockout of host cell lysosomal proteins LAMP-1 and 2 dramatically affected invasion. Likewise, triggering of lysosomal exocytosis and lysosome-dependent plasma membrane repair by low doses of streptolysin-O dramatically increased parasite entry. Together our results show that *L.amazonensis* promastigotes are able to take advantage of calcium-dependent lysosomal exocytosis and lysosome-induced endocytosis to invade and persist in non-phagocytic cells.

P1705/B844

***Trypanosoma Cruzi*-Induced Epithelial Turnover in the Placenta Might Be Mediated by the Micro Rnas 19b-3p and 515-5p.**

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Trypanosoma cruzi (*T. cruzi*), a protozoan parasite, is the causative agent of Congenital Chagas Disease[1]. For this infection to be successful, the parasite must cross the placental barrier and evade the host immune system. We have previously proposed that cellular proliferation, differentiation and apoptosis of the trophoblast (Trophoblast Turnover) can be considered as an antiparasitic local response. As most of the cellular processes, the trophoblast turnover is also regulated post-transcriptionally by small non-coding RNAs inlcuding miRNAs. However, if the *T. cruzi*-induced trophoblast turnover, and particularly the trophoblast differentiation, is mediated by miRNAs is currently unknown. MiR-515-3p and miR-19b-3p regulates trophoblast differentiation by hGCM1 gene repression. The aim of the present study was to determine if the expression of miR-515-3p, miR-19b-3p and their target gene changes in presence of *T. cruzi*. Human placental explants (HPE) were incubated in presence and absence of *T. cruzi* (Y strain) (10^5 trypomastigotes/ml) or Foskolin (100 uM) during 2 hours. MiR-515-3p and miR-19b-3p expression was further analyzed by real-time PCR by the comparative Control (ΔΔCt) method and hGCM1 expression was determined by Western blotting. *T. cruzi* decreases miR-19b-3p and miR-515-5p levels and increases hGCM1 protein expression in HPE. We conclude that the *T. cruzi*-induced trophoblast differentiation is regulated, at least partially, by miR-515-3p and miR-19b-3p. References: 1) Kemmerling U. et al. Congenital Transmission of Trypanosoma cruzi: a Review About the Interactions between the Parasite, thePlacenta, the Maternal and the Fetal/Neonatal Immune Responses. Front Microbiol. 2019 Aug 14;10:1854. doi: 10.3389/fmicb.2019.01854. 2) Liempi A. et al. A local innate immune response against Trypanosoma cruzi in the human placenta: the epithelial turnover of the trophoblast. Microb Pathog.

2016 Oct;99:123-129. doi: 10.1016/j.micpath.2016.08.022. Acknowledgements: ERANET-LAC grant ELAC2014/HID-0328 (to UK) and “Fondo Nacional de Desarrollo Científico y Tecnológico” (FONDECYT, Chile) grants 1190341 (to UK) 1170126 (to JM) and 3180452 (to ChC)

P1706/B845

Defining the Functional Secretory Proteome of *Toxoplasma during* Host Cell Invasion.

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Apicomplexans, including *Toxoplasma gondii*, are obligate intracellular parasites that rely on active invasion of host cells for their survival and replication. Secretion of proteins from specialized organelles called micronemes is essential for parasite motility, attachment to, and invasion of host cells, yet most characterized microneme proteins are dispensable for growth in human fibroblasts, suggesting extensive redundancy and/or context-specific functions. Recent identification of several microneme proteins with novel domains indicates that our current understanding of micronemes is incomplete. To define and characterize their contents, we affinity purified intact micronemes and performed quantitative mass spectroscopy. We identified most known microneme proteins in addition to many uncharacterized proteins with unusual domain architectures. We are currently employing biochemical and genetic approaches to characterize the roles of these novel microneme proteins in host cell recognition and invasion. To systematically dissect functional relationships within the microneme proteome, we have also developed a CRISPR-based screen to assess all pair-wise deletions. This genetic interaction map will define functionally related proteins and probe the phenotypes of redundant components. By defining and characterizing the microneme proteome, our results will elucidate fundamental principles of host-parasite interactions during the crucial step of invasion.

P1707/B846

How Do Malaria Parasites Break Two Membranes to Egress from Erythrocytes?

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Asexual replicative cycle of *Plasmodium falciparum* within human erythrocytes causes malaria. The parasite, a single cell organism, isolates itself from the host cytoplasm by a parasitophorous vacuolar membrane (PVM), thus both PVM and erythrocyte membrane must be breached by the time of parasite egress to initiate a new replicative cycle. Parasites developed a highly regulated parasite egress program that relies on a set of kinases and proteases and is regulated by intracellular messengers such as parasite [Ca^{2+}_{free}]. Spatial and temporal coordination of this mechanism is critical to define the targets of the different molecules of the egress program. We used *P. falciparum* with differential fluorescence labeling of the PVM and PV lumen. The natural parasite egress from erythrocytes was followed by employing time-lapse super- resolution fluorescence confocal microscopy and scanning differential interference contrast microscopy. A new obligatory stage in egress was discovered: rounding of the parasitophorous vacuole minutes before egress. Application of known inhibitors of parasite egress allowed us to clearly link sequential, morphologically definable stages of the egress pathway. The first event in the egress program was vacuole rounding, lasted approximately 1.5 minutes and controlled by parasite intracellular calcium. The next step, PVM rupture, was under control of PfPKG and SUB1 and

characterized by a progressive pattern of membrane deterioration over the following ~6 minutes up to parasite egress time. PVM rupture leads to release of vacuolar contents and initiated red blood cell membrane distortion, lasting until the last minute before egress. The newly formed parasites then mobilizes and the red cell permeabilize just before the cascade of events culminates in egress. Erythrocyte membrane rupture is controlled by cysteine proteases, possibly of parasite origin. Taken together, dissection of the membrane degradation events gives us a framework to classify yet undiscovered processes leading to parasite egress from erythrocytes.

P1708/B847

Observation of Cholesterol Sorting in *P. Falciparum* -erythrocytes by 3D Label Free Holotomographic Imaging.

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Cholesterol is essential for the growth and survival of human malaria parasite, *Plasmodium falciparum,* that is the globally important and frequently deadly for young ages. *P. falciparum* lacks a cholesterol synthesis pathway and patients infected with it often have low blood cholesterol and lipoprotein levels. We speculated that *P. falciparum* acquires cholesterol from its external environment. However, it is not completely known from where the source of cholesterol is taken up by parasitized erythrocytes, and how it crosses multiple membranes and enter parasites. To answer these questions, we used different inhibitors; statin, ezetimibe and fibrate, and investigated the effects of those reagents on *P. falciparum* growth in erythrocyte stages with and without existence of hepatocytes. The growth rate of *P. falciparum* was inhibited under the presence of ezetimibe but without hepatocyte. In co-culture system, statin strongly facilitated a better parasite growth than *P. falciparum* alone. To observe cholesterol trafficking in live *P. falciparum* in erythrocytes, we used 3D holotomographic microscopy, which mapped high refractive index for individual cellular components. After *P. falciparum* initially captured free and/or inner erythrocyte membrane-derived cholesterol, we found that the budding lipid membranes elongated and/or migrated into the cytosol and eventually fuse with the parasitophorous vacuole membrane (PVM). Under treatment of ezetimibe, membrane cholesterol accumulated in cytosol of parasitized erythrocytes and did not reach parasite. It is known that the ezetimibe targets Niemann Pick C1 like 1 (NPC1L1) in human and recent study reported that *P. falciparum* has Niemann-Pick type C1-related protein (PfNCR1). Taken together this report and our data, this study suggests; (1) *P. falciparum* imported cholesterol from extracellular environment. (2) Cholesterol is transported as a part of membrane in erythrocyte cytosol. (3) Cholesterol was taken up at the PVM by membrane fusion. (4) Cholesterol finally reaches to parasite via PfNCR1 or homology to NPC1L1. This cholesterol sorting pathway also involves the possible membrane recycling system between PVM and erythrocyte membrane. With few effective antimalarial drugs and no versatile vaccines, cholesterol transport in *P. falciparum* may become an interesting investigation target.

P1709/B848

Battle for Iron between *Leishmania* and Its Host Macrophages: Role of Phagosomal Iron Transporter Nramp1.

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Iron is critical for the proliferation of the protozoan parasites, *Leishmania* which infect and survive within the phagolysosomal compartment of mammalian macrophages. The underlying mechanism by which *Leishmania* manipulates the iron regulatory mechanism of macrophages to acquire iron for its own survival is still not clear. Natural resistance associated macrophage protein 1 (Nramp1) is an iron transporter which is expressed in the phagosomal compartment of macrophages. This protein was originally discovered because of its association with the host resistance against various intracellular pathogens, including *Leishmania*. However, the exact mechanism by which activity of Nramp1 determines the pathogenesis of *Leishmania* is yet to be discovered. Thus, the aim of this current study is to understand how *Leishmania* modulates macrophage Nramp1 to acquire iron within phagolysosomal compartment. To evaluate this J774A.1 and peritoneal macrophages of BALB/c mice has been infected with *Leishmania major* (*L. major*) and the expression level of Nramp1 was determined using Western blot and qRT-PCR and compared between uninfected and infected cells in a time dependent manner. Altered localization of the protein during infection has been studied using immunofluorescence. The total intracellular and phagosomal iron content of uninfected and infected macrophage cells was measured using ferrozine based assay. Moreover, pharmacological inhibition study has been performed to check whether Nramp1 follows proteasomal degradation pathway during *L. major* infection. Our results show infection of macrophage cells with *L. major* temporally modulates the expression of Nramp1 at the protein level without effecting its transcription. Interestingly, Nramp1 is largely present in the phagosomal compartment of uninfected macrophages but it moves to phagolysosomes during infection. Further, our results clearly demonstrate during infection downregulation of Nramp1 significantly increases the total as well as phagosomal iron content of macrophages. This increased level iron due to the loss of Nramp1 stimulates the replication of the parasite within infected macrophages. Importantly, we have established that degradation of Nramp1 during infection follows the proteasomal pathway. Collectively, this is the first report which provides direct evidences of Nramp1 to be an iron exporter and explains how *Leishmania* parasites impair the iron homeostasis of its host macrophages orchestrating the inhibition of Nramp1 for its own survival within phagolysosomes.

P1710/B849

Ire1 Activation Leads to *Toxoplasma*-infected Cell Migration.

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Homeostasis of the endoplasmic reticulum (ER) is maintained by regulation of the unfolded protein response (UPR). The UPR is induced by three ER-resident proteins, ATF6, PERK, and IRE1, which each sense perturbations in protein folding in the ER. Induction of the UPR sensory proteins trigger

transcriptional and translational modes of gene expression that serve to expand the ER processing capacity and restore homeostasis. Calcium plays an essential role inside the ER, as calcium imbalance is suggested to alter protein folding and activate the UPR. The intracellular apicomplexan parasite *Toxoplasma gondii* manipulates its infected host cells, using some as "Trojan Horses" to disseminate throughout the body. In the present study, we addressed the mechanisms by which *Toxoplasma* strategically alters ER function and protein homeostasis in host cells, thereby enhancing migration of infected cells. Our results show that association of the parasitophorous vacuole with the host ER increases calcium levels in the host cytosol, leading to activation of IRE1. It has been reported that IRE1 can modulate actin remodeling by binding filamin A, resulting in expansion of processing capacity of the ER. Our results demonstrate that *Toxoplasma* induces IRE1 dimerization leading to an increase of interaction between IRE1 and filamin A, resulting in high levels of cell migration. Our findings identify the underlying mechanisms used by *Toxoplasma* to induce dissemination of infected cells. These findings provide new insights into strategies for treatment of toxoplasmosis.

P1711/B850

Short, Minimal Viral Fusogens Hijack the Actin Cytoskeleton to Drive Cell-cell Fusion.

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Cell-cell fusion is essential for fertilization and tissue development in metazoans, and it is sometimes used by some viruses to form pathological syncytia. Cell-cell fusion is typically driven by fusogenic membrane proteins with tall (>10 nm) ectodomains that are similar to fusogens involved in enveloped viral entry. Their height likely enables them to bridge the gap between the densely-crowded plasma membranes and anchors into the opposing membrane. These fusogens then fold back to bring the two membranes into close contact to drive fusion. However, some fusogenic proteins, such as the non-structural fusion-associated small transmembrane (FAST) proteins from orthoreovirus and aquareovirus, have surprisingly short ectodomains (<2 nm) that reside within the repulsive hydration barrier that prevents membranes from coming in close contact. Yet, expression of FAST proteins in cells is sufficient to drive fusion with naïve neighboring cells, raising the question of how they bring plasma membranes in close contact for fusion. We found that p14, the reptilian reovirus FAST protein, hijacks the actin cytoskeleton to drive cell-cell fusion. Using bioinformatics, in vitro kinase and motility assays, splitYFP cell-cell fusion assay, fluorescence imaging and molecular biology techniques, we found that a tyrosine in the disordered cytoplasmic domain of p14 is important for fusion and is phosphorylated by c-src kinase. Upon phosphorylation, p14 binds to an adaptor protein, Grb2, and subsequently binds to N-WASP to nucleate branched actin assembly. This molecular pathway can be replaced by directly coupling p14 to actin assembly by using a 47-residue peptide to relieve the auto-inhibition of N-WASP. We propose that actin assembly coupled to the cytoplasmic tail of p14 provides localized force generation necessary to overcome the repulsive hydration barrier and to bring the plasma membranes in close contact so that fusion can occur. To investigate if other members of the FAST family also hijack the host actin cytoskeleton to drive cell-cell fusion, we used bioinformatics to screen all known FAST proteins for binding motifs to adaptor proteins. Interestingly, while none contained the same Grb2-binding motif found in p14, other actin-related adaptor proteins were predicted. Preliminary evidence suggests that other FAST proteins could couple to the actin cytoskeleton using these adaptor proteins to drive cell-cell

fusion, suggesting a conserved mechanism. This work reveals that overcoming energetic barriers to cell-cell fusion does not require conformational changes of tall fusogens but can instead be driven by locally harnessing the actin cytoskeleton.

P1712/B851

Flaviviruses Exploit Src-family Kinases to Orchestrate Secretion of Progeny Virions.

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Among the various host cellular processes that are hijacked by flaviviruses, very few mechanisms have been described with regard to viral secretion. Here we investigated how flaviviruses exploit the Src family kinases (SFKs) for transport and release through the host secretory pathway. We isolated three members of the SFK family - Src, Fyn and Lyn - that were activated upon Dengue and Zika infection, and also during secretion of the corresponding virus like particles (VLPs). Inhibiting their activity by pharmacological means or individual gene-depletions blocked virus production. Lyn-depletion displayed the most significant defect; we therefore generated $Lyn^{-/-}$ cells using CRISPR/Cas9 and reconstituted with its wild-type and mutant variants. Virus secretion was significantly impaired in $Lyn^{-/-}$ cells, and was rescued when reconstituted with wild-type Lyn, but not a kinase- or palmitoylation-deficient Lyn mutant. Using biochemical and imaging analyses, we established that Lyn, via its palmitoylation-dependent membrane association, triggered post-Golgi virus transport in Rab11 and Transferrin receptor positive vesicles, indicative of secretory exosomes. In the absence of Lyn activity or its aberrant membrane association, virions were sorted into the lysosomal pathway for degradation. Interestingly, Lck, a Lyn-homologous kinase, could rescue this defect in the $Lyn^{-/-}$ cells when expressing the wild-type, but not its palmitoylation-deficient mutants, indicating that members within SFK subfamilies equipped with similar biophysical properties can compensate for this mechanism of virus secretion.

P1713/B852

Employing a Cardiotropic Mouse Adenovirus to Model Acute Viral Myocarditis and Investigate Mechanisms of Arrhythmogenesis.

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Cardiovascular disease remains the leading cause of death in the United States with up to 42% of sudden cardiac death in young adults caused by myocarditis. Subcellular remodeling of the cardiomyocyte occurs during cardiomyopathy with disruptions in gap junction-mediated electrical coupling known to lead to fatal arrhythmias. The primary cardiac gap junction protein, connexin43 (Cx43), propagates innate and adaptive antiviral immune responses, leading us to hypothesize perturbations in gap junction function during active viral infection contribute to sudden cardiac death in

young adults. Adenovirus is a major etiological agent of viral myocarditis but our understanding of adenoviral infection in the heart is lacking due to viral host species specificity. Moreover, data on late-stage illness dominate in the clinic and laboratory with a resulting paucity of research focusing on mechanisms of acute viral myocarditis, and the effect of active adenoviral infection on cardiomyocyte function and arrhythmogenesis prior to induction of the immune response. Currently, Coxsackievirus and parvovirus predominate in modeling viral myocarditis but these pathogens are molecularly distinct from adenovirus in mechanisms of replication and cardiac pathology. Mouse adenovirus Type-3 (MAdV-3) was previously identified to be cardiotropic, therefore, we hypothesize that MAdV-3 can be used to model acute adenoviral myocarditis and induces an arrhythmogenic substrate. C57BL/6 adult male mice were infected via retro-orbital injection of MAdV-3 with viral tissue tropism and cardiac function assessed 7 days post-infection. Our results confirm MAdV-3 is cardiotropism by quantitative PCR of viral genomes across tissues. No significant changes in histopathology or cardiac function by echocardiography were detected in infected hearts as well so no apparent immune cell infiltration. Molecular assays, however, reveal alterations in Cx43 by western blot and immunofluorescence indicative of arrhythmogenic subcellular alterations during MAdV-3 infection. Current work includes *ex vivo* pacing of Langendorff-perfused hearts to measure conduction velocity and susceptibility of arrhythmogenesis. Taken together, our data confirm MAdV-3 as a significant and relevant model of acute adenoviral myocarditis in which antiviral and anti-arrhythmia therapeutic strategies can be developed and tested.

P1714/B853

The Intrinsic Link between Metabolic and Antiviral States of the Cell.

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Acute treatment of cells with Rapamycin promotes gene delivery by facilitating lentiviral vector entry into cells. We recently showed that mTOR inhibitors lower an intrinsic antiviral barrier mediated by the interferon induced transmembrane proteins (IFITMs), in particular IFITM3. Rapamycin-mediated degradation of IFITM3 requires endosomal trafficking, ubiquitination, endosomal sorting complex required for transport (ESCRT) machinery, and lysosomal acidification. Lentiviral transduction of diverse human and murine cells, including CD34+ hematopoietic stem progenitor cells and fibroblasts, was enhanced following acute Rapamycin treatment and enhancement was diminished upon *IFITM* knock-down or knock-out. In addition to enhancing infection by lentiviral vectors, we found that Rapamycin increased infection of replication-competent Influenza a virus by more than 10-fold. We have subsequently shown that rapamycin downregulates interferon-induced IFITM3 in the microglial cell line CHME and in monocyte-derived macrophages, which results in increased permissiveness to HIV-1 infection. Here, we describe our efforts to characterize the signaling pathway(s) that result in the negative regulation of IFITM3 during mTOR inhibition. We found that mTOR complex 2 (mTORC2) is the likely complex controlling IFITM3 levels in cells because cell starvation had minimal effect on IFITM3 while it was downregulated following knockdown of Rictor, an mTORC2 component. This result suggests that mTORC2 activity promotes the stability and function of IFITM3, linking a central metabolic regulatory network to the cell-intrinsic antiviral response. Interestingly, we found the knockdown of IFITM3 in HeLa cells resulted in decreased levels of p-Akt Ser473, suggesting that IFITM3 positively regulates Akt activation. This finding suggests a functional interdependence of IFITM3, Akt, mTORC2. Our data may suggest that 1) IFITM3 promotes Akt function by positively regulating mTORC2 signaling, and 2) a negative feedback mechanism exists which results in IFITM3 degradation following mTORC2

inactivation. We are now in the process of identifying other cellular factors that populate these interconnected pathways. Future work will include how IFITM3 influences Akt signaling during oncogenesis, since the upregulation of both are associated with cancers. Lastly, mechanistic studies of rapamycin and other mTOR inhibitors are important because these clinically important drugs, while beneficial in the setting of gene therapy *ex vivo*, may be deleterious to antiviral immunity in *vivo*.

P1716/B855

A Peptide Derived from Beclin1 Promotes Lentivirus Infection of Diverse Cell Types by Promoting Virus-cell Adhesion and Fusion.

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The development and use of transduction enhancers can facilitate the infective potential of gene delivery vectors, such as modified lentivirus. We previously found that a Tat-Beclin1 fusion peptide, which consists of the cell-penetrating peptide Tat from HIV-1 and the evolutionarily conserved domain (ECD) of Beclin1, potently enhances VSV-G-mediated transduction of CD34+ HSC by lentiviral vectors. Here we report that Tat-Beclin1 selectively promotes transduction mediated by some, but not all, viral envelope glycoproteins in a cell-dependent manner. These results indicate that enhancement of infection by Tat-Beclin1 involves an interaction between viral and cellular factors. Notably, Tat-Beclin1 increased infection by replication-competent HIV-1 in permissive cells but had no effect on Influenza a replication. To uncover the mechanism of action, we took advantage of a single residues mutation (F207S) in Tat-Beclin1 that completely abrogates its ability to promote infection. It has previously shown that the ECD of Beclin1 exhibits membrane binding activity. By studying the ability of WT and mutant peptides to associate with cells, we found that F207S prevents efficient cell association and endocytic uptake. Thus, the ability of Tat-Beclin1 to promote virus infection is linked to its ability to associate with cells. Next, we focused on the stage of the virus infection cycle that is promoted by Tat-Beclin1. We found that Tat-Beclin1 promotes the adhesion of virus to the surface of cells, which subsequently promotes the steps of virus-cell fusion and transduction. We are now in the process of identifying the membrane lipids that are preferentially targeted by Tat-Beclin1, by measuring in vitro binding between Tat-Beclin1 and liposomes of different compositions. Overall, our results reveal the potential of a Beclin1-derived peptide in gene therapy applications and further identify cell surface lipid composition as a determinant for HIV-1 entry into human cells.

P1717/B856

NUDT21 Links Cytoplasmic Stress Granules to Mitochondrial IPS-1 and Activates Host Defense Responses Against Viral Infection.

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Host cells recognize viral RNAs as “nonself” and trigger anti-viral responses to eliminate invading pathogens. RIG-I like receptors (RLRs) recognize viral RNA in cytoplasmic anti-viral stress granule (avSG), and subsequently activate the mitochondrial adaptor protein interferon-beta promoter stimulator 1 (IPS-1). However, it is not fully understood how RLRs in avSG can activate mitochondrial IPS-1 although they are spatially segregated. In this study, we revealed that Nucleoside diphosphatelinked moiety X (Nudix)-type motif 21 (NUDT21), an RNA-binding protein that regulates alternative polyadenylation in the nucleus, physically associates with IPS-1 and mediates its association with RLRs and activation of its

antiviral functions. We found that a fraction of NUDT21 was localized to mitochondria and, in response to transfection of a viral RNA mimic poly(I:C), it became co-localized to avSG. A fraction of IPS-1 was also found to localized to avSG in response to poly(I:C) transfection, and importantly, NUDT21 was required for this localization change of IPS-1. Furthermore, our data indicated that NUDT21 promotes efficient induction of type I interferons in response to RNA viral infection. These results together suggest that NUDT21 links cytoplasmic RLRs to mitochondrial IPS-1 and activates host defense responses against viral infection.

Defining Therapeutic Targets and New Therapeutics 2

P1718/B858

Paneth Cell Dysfunction in Pathophysiology of Nonalcoholic Steatohepatitis.

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Background: the prevalence of nonalcoholic steatohepatitis (NASH) is rapidly increasing worldwide. Because fibrosis, a key aspect of the pathophysiology of NASH, plays a crucial role in causing liver cirrhosis and cancer, NASH has become a global health problem. The pathophysiological mechanisms of NASH is not well understood, but emerging evidence indicated involvement of dysbiosis, an imbalance of intestinal microbiota as well as increased intestinal permeability (IP) in NASH, implicating the gut-liver axis. Paneth cells in small intestinal crypts secrete granules containing α-defensins and regulate the intestinal microbiota by potent microbicidal activities against pathogens and less activity against commensal bacteria. Paneth cells further constitute the stem cell niche and contribute to intestinal homeostasis. Thus, we hypothesize that Paneth cell dysfunction is involved in the onset of NASH via dysbiosis and increased IP. **Objective:** We aim to clarify the relationship between disruption of intestinal homeostasis due to Paneth cell dysfunction and onset of NASH. **Methods**: C57BL/6 mice were fed a choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD) for 12 weeks (w) to induce NASH or a standard diet, and were assessed at 3, 6, and 12 w. Secreted α-defensin cryptdin-1 was measured in fecal pellets by ELISA. Paneth cell morphology was analyzed by immunohistochemistry and transmission electron microscopy. The intestinal microbiota was analyzed by 16S rRNA gene sequencing. Serum zonulin, a biomarker for IP, was measured by ELISA. To determine bacterial translocation, homogenized spleen was plated on LB plates and the number of colonies was counted. **Results:** Liver fibrosis was observed in CDAHFD-fed mice slightly at 3 w and progressed gradually over 12 w. α-Defensin secretion was decreased significantly at 3 w and throughout until 12 w. Morphological analysis of Paneth cells revealed the presence of aberrant secretory granules and disruption of endoplasmic reticulum structure at 3 w, evidence of impaired granule secretion and ER stress. CDAHFD-fed mice showed dysbiosis with significantly decreased diversity and altered the composition, increased serum zonulin levels, and bacterial translocation to the spleen. **Conclusion:** These results indicate that along with reduction of Paneth cell α-defensins, dysbiosis accompanied by increased IP occurred in CDAHFD-fed mice from early on, and further suggest Paneth cell dysfunction contributes to pathophysiology of NASH via the gut-liver axis.

P1719/B859

Hepatokine Induction by Colchicine Prevents Systemic Inflammation Via Activating PTPN6 Inhibitory Signaling.

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Inflammation is a protective response to challenges including infection, injury, and tissue stresses. However, failure to resolve inflammation could lead to various diseases. The liver controls tissue metabolism and energy homeostasis by releasing secretory proteins called hepatokines. It is not clear whether liver-hepatokine-leukocyte communication contributes to inflammation regulation. Colchicine, an anti-microtubule and anti-inflammatory medication, is frequently prescribed for gout, Familial Mediterranean Fever, pericarditis, and is being clinically tested for a wide spectrum of diseases. Yet, its usage is limited by its unclear systemic action.By measuring biomarkers of microtubule perturbation and using hepatocyte-specific siRNAs, we uncovered hepatocytes are preferentially targeted by colchicine. Colchicine depolymerizes microtubules of hepatocytes and activates the transcription factor Nrf2, which regulates expression of protective genes, through promoting P62-dependent Keap1 degradation. Targeted hepatocytes subsequently secrete a novel hepatokine, GDF15, into circulation. As a divergent member of the TGF-b family, GDF15 activates inhibitory protein tyrosine phosphatase PTPN6 and inhibits inflammation via blocking leukocyte adhesion, extravasation, and IL-1b production. Our work indicates the liver mediates the anti-inflammatory activity of colchicine and opens the possibility of using anti-inflammatory hepatokines for prevention or treatment of inflammatory diseases, starting with GDF15.

P1720/B860

Some Cellular Effects of Triphenylphosphonium Derivatives of Acetylsalicylic Acid.

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Conjugation of natural compounds and active molecules with triphenylphosphonium (TPP) group has proved to be a powerful strategy for the development of effective cellular modulators and drug candidates with various activities. In this study, the TPP conjugates of acetylsalicylic acid (acetyl phosphonioalkyl-$(CH_2)_n$-salicylates (compounds 1-6)) were synthesized and characterized. The compounds 1-6 inhibited fluorescence of 2',7'-dichlorofluorescin (diacetate) probe (DCF) upon oxidation in proportion to the alkyl linker length. Effective accumulation of the TPP- acetylsalicylic acid conjugates in bacterial and mammalian cells at micromolar concentrations was detected using DCF as an intracellular probe. Bacteriostatic and antiproliferative effects of the TPP-acetylsalicylic acid conjugates were studied with the aid of broth microdilution method and MTT assay, respectively. The TPP group provided enhanced activity of the compounds against both Gram-positive bacteria and cancer cell lines with MIC and IC_{50} values as low as c.a. 1 μM. The cellular effects of the compounds were also linker-dependent. According to dynamic light scattering technique, the selected conjugate differently affected the zeta potential of E. coli and S. Aureus after 1 h treatment of the bacteria with the compound. The results suggest potential antibacterial and anticancer activities of the synthesized TPP-acetylsalicylic acid

conjugates. In vitro mechanisms of their action are under investigation. This work was funded by the subsidy allocated to Kazan Federal University (4.5151.2017/6.7) and by the Program of Competitive Growth of KFU.

P1721/B861

Explaining the Relative Inactivation Efficiencies of FAAH Inhibitors BIA 10-2474, JNJ-42165279 and PF-04457845.

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BIA 10-2474 is a fatty acid amide hydrolase (FAAH) inhibitor which was under clinical development [1], when a phase I trial was ended prematurely in 2016, after one person passed away and four other subjects suffered severe emergent CNS adverse events. The inactivation efficiency (k_{inact}/K_I) of BIA 10-2474 was previously studied and compared to those of other FAAH inactivators (JNJ-42165279 and PF-04457845) under clinical evaluation. All three molecules are time-dependent FAAH inactivators and PF-04457845 is the most efficient one, whereas JNJ-42165279 and BIA 10-2474 present lower in vitro inactivation efficiencies. [2] Advanced computational chemistry methods were employed to study the mechanisms of enzyme inactivation induced by the three investigational drugs. Based on these results the nature of the differences in their inactivation efficiencies is discussed. The molecular interactions were studied by extended molecular docking and dynamics simulations, whereas the detailed inactivation mechanisms of the reaction with the enzyme were studied using hybrid quantum mechanics/molecular mechanics (QM/MM). In the initial step of time-dependent inactivation, the inhibitor is required to enter and explore the enzyme's active site, where non-covalent interactions will eventually steer the molecule towards a near-reactive configuration, before it may react with the catalytic triad and form an irreversible adduct. The kinetic efficiency of this initial process affects the rate of the time-dependent inhibition. Our results show that PF-04457845 is the most efficient of the three inhibitors, followed by JNJ-42165279 and by BIA 10-2474. On the other hand, our QM/MM calculations confirmed that all three inhibitors may readily react with the enzyme's catalytic triad and form irreversible adducts, even though through distinct reaction mechanisms. Overall, our results allow to conclude that the rate-limiting step of the time-dependent inactivation of FAAH by all three compounds, is the formation of a specific near-reactive configuration between enzyme and inhibitor. [1] Kiss LE, Beliaev A, Ferreira HS, Rosa CP, Bonifácio MJ, Loureiro AI, Pires NM, Palma PN, Soares-da-Silva P., 2018, ChemMedChem., 13(20), 2177-2188. [2] Bonifácio MJ, Loureiro A, Aires C, Fernandes-Lopes C, Sousa F, Palma N, Moser P, Soares-da-Silva P., 2018, Mol Biol Cell, 28, 630 (Abstract P2651).

P1722/B862

Anthrax Edema Toxin Co-opts IGF1R and EGFR Signaling to Promote Cell-cell Barrier Dysfunction.

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Cell-cell barrier dysfunction induced by *Bacillus anthracis* edema toxin (ET) is central to bacterial dissemination and lethal vascular collapse manifested during the fulminant, late-stages of infection. Available treatments including antimicrobial and early-stage antitoxin measures offer very limited protection against anthrax-induced fatality once the toxins released by the vegetative bacilli enter host cells and tissues and exert their intracellular function. Therapeutic measures that specifically mitigate

the consequences of cellular intoxication by anthrax toxins could effectively stem and potentially reverse the extreme morbidity and high fatality rates associated with systemic anthrax. This study identifies cell-based therapeutic measures that target the late-stage intracellular activities of ET and their potential to mitigate the consequences of ET toxemia. Using cell-based mechanistic studies, we uncover a critical involvement of receptor tyrosine kinases EGFR and IGF1R in promoting ET-dependent barrier disruption through effects on cellular F-actin network dynamics that are separate from, but have an impact upon, previously characterized EF-mediated inhibition of Rab11-dependent vesicular trafficking. ET rapidly trans-activates IGF1R and EGFR, resulting in the activation of downstream effectors PI3K and MEK, and subsequently a Rac1- and cofilin-dependent actin remodeling. Furthermore, we provide in *vivo* pre-clinical validation of the therapeutic potential of PI3K, MEK and Rac1 inhibitors in preventing ET-dependent edema in a mouse footpad model. In conclusion, we identify a combinatorial set of druggable cellular targets that mitigate late-stage effects of anthrax ET. Antitoxin measures targeting ET should significantly enhance patient survival during advanced, systemic stages of the disease.

P1723/B863

Claudin Targeting by Modular Biologicals Based on *Clostridium Perfringens* Enterotoxin.

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Clostridium perfringens enterotoxin (CPE) is a bacterial, ß-pore-forming toxin. It binds to the extracellular domain of some claudins, e.g. claudin-3 (Cldn3), -4 or -7. CPE-insensitive claudins include Cldn1, -2 and -5. Upon binding to its receptors via its C-terminal domain (cCPE), the CPE-claudin complexes rapidly oligomerizes and the N-terminal domains form a ß-pore in the plasma membrane leading to Ca^{2+}-influx induced cell death. Structure-guided mutagenesis of full length CPE and the non-toxic cCPE enabled targeting of otherwise CPE-insensitive claudins. The mutations S305P/S307R/S313H facilitates binding to Cldn1-9, whereas mutation S231R/S313H improved Cldn1-binding properties. Here we present different applications of modified (c)CPE (i) Cldn1 and CD81 constitute an essential entry factor for hepatitis C virus (HCV). We showed that, in contrast to cCPEwt, cCPE-S305P/S307R/S313H efficiently inhibits HCV infection of human Huh7.5 hepatoma cells. Homology models suggest that cCPE-SSS masks residues in the extracellular domain of Cldn1 which are required for interaction between Cldn1 and CD81. FRET analysis in HEK-293 cells co-transfected with CFP-Cldn1 and YFP-CD81 affirms this theory.(ii) Various cancer entities have deregulated and delocalized claudin expression. Cldn1 expression is upregulated in a broad spectrum of cancers, e.g. thyroid and lung cancer. Substitution S231R/S313H enabled targeting of Cldn1-overexpressing papillary thyroid carcinoma cells (K1) and improved that of non-small cell lung cancer (PC-9). Cell line-derived xenografts of PC-9 and K1 tumors had a markedly reduced growth rate after intra-tumoral injection with CPE-S231R/S313H. Histological and immunofluorescence staining revealed that this effect is due to an increase in necrotic tissue and reduced proliferative activity of the cancer cells.(iii) Tight junctions in the *stratum granulosum*,

containing Cldn1 and -4, contribute to the epidermal barrier. Treatment of reconstructed human epidermis with cCPE variants weakened the epidermal barrier as suggested by transepithelial resistance and Lucifer Yellow flux measurements. A transient opening of the epidermal barrier might improve transdermal drug delivery.In sum, we were able to show that directed mutagenesis of cCPE and CPE facilitates targeting of otherwise CPE-insensitive claudins. This opens up new possibilities of therapeutic application of cCPE and CPE where a claudin-directed approach is advantageous.

P1724/B864

***In Vitro* Effects of Fluoroacetate and Fluorocitrate Toxicity.**

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Despite its early application as a rodenticide, fluoroacetate, an acutely toxic metabolic poison, is restricted in the United States due to comparable lethality to other mammals, including humans. Although the mechanism of action is known, no countermeasures have been identified, and accidental ingestion by humans commonly results in death. Once internalized, fluoroacetate undergoes a “lethal synthesis” and is converted to fluorocitrate, a tightly binding competitive inhibitor of mitochondrial aconitase, effectively halting mitochondrial respiration. This disruption of the citric acid cycle leads to mitochondrial dysfunction with physiological symptoms including cardiac dysfunction, neurological effects, and renal failure. By characterizing the cellular effects of the toxicant fluoroacetate, and the toxin fluorocitrate, we can more effectively evaluate possible courses of treatment and better understand how to defend against metabolic poisons that target the citric acid cycle. Using an in *vitro* model, we have increased understanding of fluoroacetate toxicity. Since only a small amount of fluoroacetate is converted to fluorocitrate, the toxicant was not an effective compound to study the effects on human cells in *vitro*. However, in response to fluorocitrate, mitochondrial respiration in cardiomyocytes showed concentration- and time-dependent responses with decreases in maximal and spare respiratory capacity ($p < 0.01$), thereby generating a baseline against which to evaluate potential medical countermeasures. Multiple classes of therapeutics were analyzed: antioxidants, for their ability to minimize cellular damage; TCA substrates, which enter the citric acid cycle downstream of aconitase, in an effort to rescue cellular respiration; and mediators of alternative energy pathways for their ability to improve cellular viability with the goal of allowing an organism the opportunity to clear the toxin over time. In hepatocytes, the antioxidants glutathione and N-acetyl-L-cysteine and citric acid cycle substrates D,L-isocitric acid and glycerol monoacetate showed the greatest promise. Together, these analyses offer a comprehensive view of the cellular effects of fluoroacetate/fluorocitrate intoxication and provide critical direction to the search for effective medical countermeasures to this and other metabolic poisons.

P1725/B865

Binding and Inactivation Mechanisms of the Fatty Acid Amide Hydrolase Inhibitor BIA 10-2474.

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Several inhibitors of fatty acid amide hydrolase (FAAH) have been developed as a strategy for the treatment of various nervous system disorders [1]. In 2016, during a phase I clinical trial with the FAAH

inhibitor BIA 10-2474, five subjects suffered severe emergent CNS adverse events, with one fatal outcome. The mechanism of FAAH inactivation by BIA 10-2474 was wherein studied with atomistic resolution using molecular docking, molecular dynamics simulations and hybrid quantum mechanics/molecular mechanics (QM/MM) simulations. These results were also compared with the catalytic mechanism of the FAAH natural substrate oleamide. Our results are consistent with the observed time-dependent FAAH inactivation by BIA 10-2474 [2]. Upon binding to the active site, the inhibitor eventually adopts a low energy near-reactive configuration, where the urea moiety is effectively cleaved by the enzyme's catalytic triad (Ser-*cis*Ser-Lys) and forms a stable carbamoyl adduct. The QM/MM results demonstrate that the hydrolysis of this adduct, as well as the reverse reaction have very high activation free energies and are not possible under physiological conditions, therefore leading to the irreversible inactivation of the enzyme. The results also reveal the important role played by FAAH Thr236 in the reaction with BIA 10-2474, which is specific of FAAH and is not present in other serine hydrolases. It forms a hydrogen bond with the imidazole nitrogen of the inhibitor, and helps lowering the activation free energy of the first step of the reaction, by pre-orienting and stabilizing the inhibitor in a near-reactive configuration. Finally, the comparison with the catalytic mechanism of the natural substrate oleamide also reveals key differences between substrate and inactivator. The inhibitor shows a faster and more effective reaction with the enzyme to form a stable and irreversible carbamoyl adduct, whereas the substrate forms a transient acyl adduct that can be readily hydrolyzed by water to complete the catalytic cycle and regenerate the active enzyme. 1. Kiss LE, Beliaev A, Ferreira HS, Rosa CP, Bonifácio MJ, Loureiro AI, Pires NM, Palma PN, Soares-da-Silva P., 2018, ChemMedChem. 13(20), 2177-2188. 2. Bonifácio MJ, Loureiro A, Aires C, Fernandes-Lopes C, Sousa F, Palma N, Moser P, Soares-da-Silva P., 2018, Mol Biol Cell, 28, 630 (Abstract P2651).

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Identification of Cathepsin D as a Plasma Biomarker Candidate for Alzheimer's Disease.

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Although Alzheimer's disease (AD) is the most common neurodegenerative disease, there are still no drugs available to treat or prevent AD effectively. However, efforts to develop early diagnostic ways should also be made along with the development of treatment of AD for appropriate medical intervention from the early stage of the disease. Here, we examined changes in levels of selected proteins implicated in the pathogenesis of AD using plasma samples of control subjects and patients with cognition impairment. To precisely categorize the disease, fifty-six participants were examined with clinical cognitive tests, amyloid PET scan and white matter hyperintensities were scored by magnetic resonance imaging. Plasma cathepsin D levels of the subjects were examined by immunoblotting and ELISA and compared between groups. Correlation of plasma cathepsin D levels with AD-related factors and clinical characteristics was examined by statistical analysis. By analyzing quantitative immunoblot and ELISA, we found that the plasma level of cathepsin D, a major lysosomal protease, was significantly decreased in the group with amyloid plaque deposition at the brain compared to the control group. The level of plasma cathepsin D was negatively correlated with CDR-SB scores. These results suggest that the plasma cathepsin D level could be developed as a diagnostic biomarker candidate of AD. Results of this study also suggest that lysosomal degradation activity could be associated with the onset or progression of AD.

P1727/B867

Brain Somatic Mutations in Mtor Reveal Translational Dysregulations Underlying Intractable Focal Epilepsy.

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Brain somatic mutations confer genomic diversity in the human brain and cause neurodevelopmental disorders. Recently, brain somatic activating mutations in *MTOR* have been identified as a major etiology of intractable epilepsy in patients with cortical malformations. However, the molecular genetic mechanism of how brain somatic mutations in *MTOR* causes intractable epilepsy has remained elusive. In this study, translational profiling of intractable epilepsy mouse models with brain somatic mutations and genome-edited cells revealed a novel translational dysregulation mechanism and mTOR activation-sensitive targets mediated by human *MTOR* mutations that lead to intractable epilepsy with cortical malformation. These mTOR targets were found to be regulated by novel mTOR-responsive 5′ UTR motifs, distinct from known mTOR inhibition-sensitive targets regulated by 5′ TOP motifs. Novel mTOR target genes were validated in patient brain tissues, and the mTOR downstream effector eIF4E was identified as a new therapeutic target in intractable epilepsy via pharmacological or genetic inhibition. We show that metformin, an FDA-approved eIF4E inhibitor, suppresses intractable epilepsy. Altogether, the present study describes translational dysregulation resulting from brain somatic mutations in *MTOR*, as well as the pathogenesis and potential therapeutic targets of intractable epilepsy.

P1728/B868

Identifying Loss-of-function Mutants That Confer Resistance to Antidepressants in *Saccharomyces Cerevisiae*.

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Serotonin transporters are the primary targets of one of the most highly prescribed classes of antidepressants, the selective serotonin reuptake inhibitors (SSRIs). Yet, it is unknown what additional biological pathways and targets SSRIs might have. To address this question, there have been several studies of antidepressants in the budding yeast, *S. cerevisiae* investigating possible “off-target” effects of SSRIs. Budding yeast is an ideal organism for “off-target” effects since they lack serotonin transporters. However, most reports have focused on loss-of-function mutations that render cells hypersensitive to SSRIs. While this work has pointed to new activities such as chromatin remodeling, it remains challenging to determine the direct target(s). We sought to complement these results by screening for mutations that confer resistance. Isolating resistant mutants has the potential to identify direct drug targets or activities required for drug toxicity, giving important insights into drug mechanism. In this work, we screened a yeast library comprised of 4,577 gene deletions for deletions resistant to fluoxetine, also known as Prozac. The primary screen identified 103 deletions that were resistant to an otherwise lethal dose of fluoxetine. We performed spot test assays for resistance to fluoxetine as well as another SSRI, sertraline (Zoloft), and a non-SSRI, amitriptyline (Elavil). We confirmed 48 gene deletions conferred some degree of resistance to at least one of the drugs. We remade 18 of the most resistant deletions to fluoxetine and observed that 10 of the *de novo* deletions displayed resistance to fluoxetine, suggesting that the majority of the 48 deletions are true positives. In addition to genes that function in vacuolar-ATPase activity, which were previously implicated, we found several genes with functions in protein homeostasis (e.g. protein degradation and synthesis). We are working toward understanding how changes in protein homeostasis confer resistance to SSRIs. We have so far tested the hypothesis

that these mutants increase drug efflux activity and found that most do not change the activity of the major efflux pump, Pdr5. Efforts are underway to characterize the physiology of cells treated with SSRIs in order to understand how protein homeostasis mutants may be able to confer resistance. This work will contribute to a better understanding of how antidepressants might act in neuronal cells by revealing new targets apart from the serotonin reuptake transporter. Considering that antidepressants are orally taken, homologous microbial targets might lead to changes in gut microbial activity and/or composition, that may play an important role in antidepressant efficacy.

P1729/B869

Lack of Association of Tnf-α Functional Variant (-308 G/A) with Turkish Schizophrenia Patients.

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Schizophrenia (SCZ) is one of the most common devastating psychiatric disorders that negatively affects the quality of life and psychosocial functions. Inflammatory markers are well-known etiological factors for psychiatric disorders, including schizophrenia. Tumor necrosis factor (TNF), encoded by TNF-α gene has an important role in the apoptotic mechanisms of autoimmune diseases. Recently, TNF-α polymorphisms and autoimmune/psychiatric disorders have been reported to be related. This study aims to determine the impact of functional variant in the TNF- α gene (-308 G/A) on the susceptibility of schizophrenia. The TNF- α gene variant (-308 G/A) were genotyped 119 individuals with SCZ patients and 94 healthy controls. Through applying salting out procedure, we extracted DNA from peripheral blood samples. These variant were analyzed using PCR-RFLP. De Finetti program and SPSS version 14.0 for Windows were used for analyzing the data. The presence of genotypes with SCZ patients were A/A 86.5%,G/A 11.7% and G/G 1.6% whereas A/A 81.9%,G/A14.9% and G/G3.2% in healty controls. No significant differences were observed between groups for TNF- α gene variant (-308 G/A) genotypes and allele frequencies in SCH patients. Our results showed that functional variant of the TNF- α gene (-308 G/A) might not play a potential role in SCZ pathophysiology. However, further studies are warranted involving a larger sample size to strengthen our findings.

P1730/B870

(-)-Epigallocatechin-3-gallate Treatment Increases Survival of Pml/rarα Mice, Reduces Leukemic Burden and Induces Cell Differentiation.

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(-)-Epigallocatechin-3-gallate (EGCG) is a gallate ester obtained by the condensation of gallic acid with the (3R)-hydroxy group of (-)-epigallocatechin. This component, extracted from green tea, has multiple effects on signal transduction pathways and enzyme activities which could enhance apoptosis and suppress of cell proliferation, invasion, angiogenesis and metastasis in cancers. This study aims to

evaluate the effect of EGCG in an experimental model of leukemia. NOD.CB17-Prkdc^{scid}/J mice received 2*Gy* irradiation followed by transplantation of leukemia cells obtained from hCG-PML-RARα transgenic mice by i.v. Injection in the caudal vein. Establishment of disease was confirmed at day 12 through presence of leukocytosis, and/or anemia, and/or thrombocytopenia, associated to the presence of blasts in blood. At 12th day, mice (n=10/group) were randomly selected to receive EGCG (25mg/kg/day) or vehicle i.p. For five consecutive days. Mice were then sacrificed and peripheral blood (PB), bone marrow (BM) and spleens were collected for flow cytometry and western blot analysis. Hematological analysis revealed that EGCG treatment reversed leukocytosis (p=0.0371), anemia (p=0.0155) and thrombocytopenia (p=0.0179) and prolonged survival of PML/RARα mice (p=0.0017). Notably, EGCG reduced leukemia immature cells (CD45$^+$CD34$^+$)(p=0.0060) and promyelocytes (CD45$^+$CD117$^+$)(p=0.0157) in BM of mice whereas increased mature myeloid cells (CD11b$^+$Gr-1$^+$)(p=0.0051), possibly by inducing cellular differentiation. These results were corroborated by the reduction in promyelocytes (p=0.0154), and the increase in neutrophils (CD45$^+$Gr-1$^+$)(p=0.0178) and monocytes (CD45$^+$CD11b$^+$)(p=0.0463) detected in PB. We then evaluated the effect of EGCG on cellular differentiation by studying degradation of PML/RARα oncoprotein. EGCG increased the percentage of cells with aggregated PML bodies stain in the BM of PML-RARα mice, suggestive of higher degradation of oncoprotein, parallel to a reduction in PIN1 expression in BM cells. Higher intracellular levels of reactive oxygen species (ROS) were also detected in leukemia immature cells (p=0.0051), promyelocytes (p=0.0271) and neutrophils (p=0.0033) of BM. These results are consistent with literature data demonstrating that the ablation of PIN1 and/or induction of ROS could trigger PML/RARα degradation. Furthermore, apoptosis was detected in spleen cells of PML-RARα mice (p=0.0197) in parallel to increased expression of BAX, reduced expression of BCL-2, and reduction of spleen weight (p=0.0085). Collectively, our results support further evaluation of EGCG in clinical trials for acute myeloid leukemia.

P1731/B871

Rac1 Stimulation of Oxidative Stress Signaling Contributes to Epithelial to Mesenchymal Transition in Ovarian Cancer Cells.

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The Rho family of small GTPases, including Rac1 and Cdc42 are often overexpressed in cancer and associated with poor patient outcomes. Elevated Rac1 activity promotes cell proliferation, quiescence, epithelial to mesenchymal transition (EMT) and transcriptional programs relevant to tumor development, progression and chemoresistance. In this study, modest (~4-fold) overexpression of Rac1 in ovarian tumor cells caused morphological changes consistent with EMT. Rac1 overexpression decreased colony number, increased colony area, and increased cell invasion through an artificial basement membrane 10-fold when compared to vector control cells. An increase in mesenchymal markers (e.g. N-cadherin and vimentin) and decrease in epithelial markers (e.g. E-cadherin, claudin) was observed as a consequence of Rac1 expression. Rac1 overexpression was associated with selective up-regulation of certain EMT transcriptional regulators with increased mRNA expression of Slug/Snai2 (8-fold) and Zeb2 (100-fold) and little impact on Snai1, Twist or Zeb1. Slug/Snai2 expression is induced by oxidative stress and treatment of six ovarian tumor cell lines with tert-butyl hydroperoxide (TBHP) to generate oxidative stress increased the expression Slug/Snai2, Snai1 and heme oxygenase-1, an indicator of oxidative stress response. Because activation of NADPH oxidases 1, 2 & 3 is dependent upon Rac1 activity thereby leading to generation of reactive oxygen species (ROS), we tested oxidative stress

signaling as a consequence of Rac1 overexpression. The basal level of ROS was elevated in cells overexpressing Rac1 compared to vector control cells as detected by the fluorescent probe Dihydroethidium. Similarly, elevated Rac1 increased expression of the oxidative stress response genes heme oxygenase-1 (17-fold) and superoxide dismutase-1 (12-fold) further supporting that Rac1 stimulated ROS signaling in these cells. We further tested the impact of Rac1 inhibition in *vivo* using R-ketorolac. An alysis of tumor tissue from mice showed decreased expression of KRT19, heme-oxygenase-1 and Slug/Snai2 in mice treated with R-ketorolac compared to placebo controls. Collectively, these findings demonstrate the functional impact of Rac1 overexpression in ovarian cancer cells and suggest that Rac1-dependent activation of oxidative stress signaling in ovarian cancer may contribute to EMT in ovarian tumors.

P1732/B872

Porcine Placental Extract Exerts Fibroblast Growth Factor Activity and Suppresses Hair Growth.

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[Objectives] the placenta is a multifaceted, transient organ expressing a variety of biologically active molecules. Its postpartum utilization for cosmetic and healthcare purposes have long been performed through topical application, subcutaneous injection or oral consumption. We previously reported that porcine placental extract (PPE) powder, manufactured through heat treatment and enzyme digestion as a cosmetic ingredient, contains biologically active substance(s) that activates fibroblast growth factor (FGF) receptors. Since FGF signalings play multiple roles in hair growth cycle regulation, we aimed at examining the effects of PPE on murine hair growth in this study. [Methods] Nine-week-old C3H/HeN male mice, with their dorsal hair follicles in telogen (resting) phase of hair growth cycle, were used. The dorsal club hairs were trimmed short, and the mice were topically given various test samples in 100 µl solution for 28 consecutive days, and the hair growth was observed and photographed. The samples tested were; phosphate buffered saline (negative control), 5% Minoxidil (positive control), 40 mg/ml Unfractionated PPE (UPPE), High-MW PPE (HPPE: MW > 10 kDa), Medium-MW PPE (MPPE: 3~10 kDa) and Low-MW PPE (LPPE: MW < 3 kDa). The hair growth was scored from the photographs. After 28 days, the mice were sacrificed, skin samples were collected and the hair follicles were morphologically observed. [Results] a significant hair-growth-suppressing effects were observed for all the PPE samples examined. UPPE showed the strongest hair-growth-suppressing effect, followed by HPPE, MPPE and LPPE in this order. Hair follicles of telogen (resting) and early anagen (growing) phases were observed in mice that received UPPE and HPPE. In MPPE- and LPPE-treated mice, hair follicles of late anagen and catagen (regressing) phases were also observed, indicating modest retardation of telogen-to-anagen transition. [Conclusions and Discussion] PPEs exert strong suppressive effects on both telogen-to-anagen transition and anagen progression. As UPPE and HPPE exert strong effects, it is likely that high-MW substances contained in PPE are responsible for this activity. This finding may suggest, or does not exclude the possibility, that one or some of the FGF receptor agonist substances are responsible for the hair-growth-suppressing activity of PPE.

P1733/B873

The Inhibition of Cxxc5-dvl Interaction Stimulates Hair Regrowth and Wound-induced Hair Neogenesis through Activation of Wnt/b-catenin Pathway.

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The Wnt/β-catenin pathway has been implicated in hair follicle development and hair regeneration in adults. We discovered that CXXC-type zinc finger protein 5 (CXXC5) is a negative regulator of the Wnt/β-catenin pathway involved in hair re-growth and wound-induced hair follicle neogenesis (WIHN) via an interaction with Dishevelled (Dvl). CXXC5 was up-regulated in miniaturized hair follicles and arrector pili muscles in human balding scalps. The inhibitory effects of CXXC5 on alkaline phosphatase activity and cell proliferation were demonstrated using human hair follicle dermal papilla cells. Moreover, CXXC5-/- mice displayed accelerated hair re-growth and treatment with valproic acid, a glycogen synthase kinase 3β inhibitor that activates the Wnt/β-catenin pathway, further induced hair re-growth in the CXXC5-/- mice. Disrupting the CXXC5-Dvl interaction with a competitor peptide activated the Wnt/β-catenin pathway and accelerated hair re-growth and WIHN. Overall, these findings suggest that the CXXC5-Dvl interaction is a potential target for treatment of hair loss.

P1734/B874

Different Protein Expression in Raw 264.7 Cell Treated with Pentoxifylline through Immunoprecipitation High Performance Liquid Chromatography.

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Pentoxifylline (PTX) is a methylxanthine derivative that have been developed as an immunomodulatory agent and an improvement of microcirculation. To reveal PTX effects as the cellular levels, protein expression profiles were analyzed in the PTX-treated RAW 264.7 cells by using immunoprecipitation high performance liquid chromatography (IP-HPLC). After treating RAW 264.7 cell with PTX at 10 μg/ml for 12, 24, and 48 hours, RAW 264.7 cells were harvested. The protein extracts were used for IP-HPLC analyze of proliferation-, epigenetic modification-, translation-, differentiation-, growth factor-, immunity-, inflammation-, p53-mediated apoptosis-, FAS-mediated apoptosis, cell protection -, antioxidant-, angiogenesis-, and osteogenesis-related protein in the macrophage. PTX was shown to inhibit cellular proliferation, apoptosis, and inflammation in RAW 264.7 cells. Conversely, angiogenesis and antioxidant activity were not significantly affected. Although RAW 264.7 cells are a macrophage cell line, PTX was expected to have an osteogenesis promoting effect, which is expected to provide an advantage in the treatment of osteonecrosis with anti-inflammatory effects IP-HPLC results indicate that PTX plays wound healing roles in RAW 264.7 cells by regulating anti-inflammation-, cell proliferation-, osteogenesis-, immunity-, and apoptosis- related proteins. The anti-inflammatory effect, anti-apoptotic effect and osteogenesis promoting effect are thought to be helpful in the treatment of osteoradionecrosis. *This research was supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Education (No. 2017R1D1A1B03036054).

P1735/B875

Inhibition of TGF-beta Signaling in a Cellular Model of Human Post-traumatic Heterotopic Ossification.

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Heterotopic ossification (HO) is characterized by the abnormal growth of ectopic bone in non-skeletal soft tissues. Previous studies have demonstrated that TGF-beta (TGFB) levels are elevated in the soft tissues of war-traumatized extremity injuries. Since TGFB mediates most of the initial inflammatory and wound healing response in the traumatized muscle bed, we hypothesized that targeted inhibition of the TGFB pathway may be able to abrogate the unbalanced fibrotic and bone forming response observed in HO. To investigate that we used primary multipotent progenitor cells (MPCs) harvested from debrided traumatized human muscle tissue that are capable of differentiating down multiple pathways, including bone and cartilage. The specimens were taken at the margin of devitalized and healthy appearing tissue and would otherwise have been discarded as surgical waste. The Walter Reed National Military Medical Center IRB waived the need for consent. MPCs treated with TGFB had increased levels of the fibrotic markers *ACTA2*, *COL1A1*, *FN1* and *SERPINE1* (4.1- to 9.5-fold) compared to control, while upon treatment with TGFB inhibitors the expression of the fibrotic markers was reduced 0.3- to 7.9-fold. Treatment of MPCs with TGFB also showed increased expression of the osteogenic regulator *CBFA1* (1.9-fold change) compared to control, while *CBFA1* induction was moderately reversed (0.1- to 0.9-fold decrease) when treated with TGFB inhibitors. At the protein level, treatment with inhibitors that block the TGFB receptor showed a dose-dependent reduction of pSMAD2 and pSMAD3 compared to control. In addition, targeted SMAD3 inhibitors had no significant effects in pSMAD2, while a dose-dependent inhibition of pSMAD3 compared to the control was observed. Total SMAD2 and SMAD3 levels were mostly not significantly affected. Finally, selected TGFB inhibitors abrogated the formation of fibrotic nodules in a model of fibrotic nodule formation using MPCs. These results demonstrate that TGFB alone is sufficient to induce a fibrotic and osteogenic phenotype in post-traumatic MPCs, demonstrating that these cells are a robust in *vitro* model for the study of HO. In addition, our results support the notion that targeted inhibition of the TGFB pathway may be a useful therapeutic strategy for post-traumatic HO patients. **Disclaimer:** the contents of this publication are the sole responsibility of the author(s) and do not necessarily reflect the views, opinions or policies of Uniformed Services University of the Health Sciences (USUHS), the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., the Department of Defense (DoD), the Departments of the Army, Navy, or Air Force. Mention of trade names, commercial products, or organizations does not imply endorsement by the U.S. Government.

P1736/B876

Pentoxifylline and Tocopherol Effect on Cortical Bone of Rat Irradiated Jaw: a Micro-Ct and Immunohistochemical Study.

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Pentoxifylline (PTX) and tocopherol (TP) combination is believed to have the effect of reducing the chronic fibrosis and induce bone healing in osteoradionecrosis (ORN) of mandible, but evidence of its effectiveness on cortical bone is lacking. This study was designed to determine effect of PTX and TP

combination on mandibular cortical bone remodeling in ORN rat model, using micro-CT and histological analysis. Forty-eight eight-week-old male Sprague-Dawley rats randomly divided into irradiated group (n=40) and non-irradiated (n=8). An imals in irradiated group were divided into 4 sub-groups, including PTX, TP, both PTX and TP and NS (normal saline). Three weeks after irradiation, mandibular posterior tooth extraction was performed and animals were sacrificed seven weeks after irradiation. The mandible was analyzed using micro-CT and histological evaluation. The alveolar bone height (ABH) and cortical bone thickness (CBT) of PTX+TP group were significantly higher than other groups. In the irradiated group, the cortical bone volume (Ct.BV) and total cortical bone surface (Ct.S) of PTX+TP group were significantly higher than those of the PTX, TP and NS groups. The average CBT (Ct.Th) of the PTX+TP group was significantly higher than that of the NS group ($p < 0.05$). In the 3D reconstructed images, there was an inadequacy in the residual volume of cortical and cancellous bone in the irradiated groups. Viable blood vessels within the Haversian canals can be observed in the PTX+TP group, and a small quantity is observable in the PTX group. Our finding demonstrated that the combination of PTX and TP improved quality and quantity of cortical bone in irradiated rat mandible, thus supporting its utility as treatment and prophylaxis agent for ORN. We observed an inadequacy in the destruction of cortical bone and cancellous bone in ORN mandible, suggesting that cortical bone could play an important role in further ORN studies. *This research was supported by Basic Science Research Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Education (No. 2017R1D1A1B04029339).

P1737/B877

Dec1-mediated Circadian Rhythm Disruption Aggravates Osteoarthritis Pathogenesis.

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Molecular circadian clock pathway including BMAL1, CLOCK, PER1/2, and CRY1/2 are involved in the most physiological and behavioral process such as the blood pressure, metabolic state, sleep/wake cycle with 24-hour oscillations. It is also known that the disruption of circadian rhythms is closely related to human health and aging. As an aging, rhythmical behaviors such as sleep/wake patterns become fragmented, and amplitude of molecular clock gene oscillations are progressively weakened. Although the effect of circadian rhythms on skeletal development and degenerative disease remains unclear, there is some evidence indicating the role of circadian rhythm on the pathogenesis of osteoarthritis (OA), the most frequent degenerative disease in the world. Given that aging is a well-known risk factor of OA, weakened oscillation of molecular clock gene pathway during aging could affect the catabolic effect on chondrocytes, a sole component of joint cartilage tissue. In this study, we investigate the link between OA and DEC1-mediated circadian rhythm disruptions. Differentiated embryo chondrocyte expressed gene 1 (DEC1) is one of the target genes regulated by core clock genes, BMAL1-CLOCK heterodimer, and also known to inhibit the activity of BMAL1-CLOCK heterodimer by binding to the E-box region of target genes promoter. For in vivo experiments, Human OA cartilage and mouse OA animal model were used. The expression of DEC1 was upregulated in cartilage tissue of OA patients. Furthermore, cartilage degradation induced by DMM surgery on mice joint was decreased in DEC1 KO mice. Moreover, adenoviral overexpression of DEC1 in the mice joint by intra-articular injection showed severe cartilage degradation. For in vitro experiments, primary chondrocyte was used. In chondrocytes, it was confirmed that the expression of DEC1 increased by treatment of IL-1β. IL-1β also caused rhythmicity disruption of clock genes including BMAL1, CLOCK, and PER2. Additionally, overexpression of DEC1 upregulated the expression levels of OA related catabolic factors, such as MMP3, MMP13, IL6,

PTGS2 and ADAMTS4, and DEC1 knockdown reduced IL-1β-induced overexpression of catabolic factors. In conclusion, our results suggest that upregulation of DEC1 cause disruption of circadian rhythms and this disruption cause degradation of cartilage by upregulation of OA-related catabolic factors.

P1738/B878

Role of Chondrocyte-derived Serotonin on Arthritic Cartilage Destruction.

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Serotonin synthesized by Tph2 in brain acts as a neurotransmitter and it synthesized by Tph1 in the periphery acts as a hormone or intracellular signaling factor. In this study, we investigated the effect of chondrocyte-derived serotonin on cartilage breakdown in osteoarthritis (OA). Tph1 was increased not only in human and mouse OA cartilage but in chondrocytes which induced arthritis-like condition. Also serotonin amount in synovial fluid was increased in OA mouse model. So we hypothesized that serotonin both intracellular and extracellular exacerbates cartilage destruction. Exogenously added serotonin did not affect gene regulation such as inflammatory factors and matrix degrading enzymes. However, serotonin treatment increased the key inflammatory genes such as Cox2, iNOS, and IL-6 as well as MMPs in the presence of IL-1β. Moreover, we found that IL-1β treatment exhibited the increased gene expression of serotonin receptors such as Htr1B and Htr2A. To investigate the molecular regulatory action modes of serotonin on the up-regulation of inflammatory gene expression, specific antagonist against Htr1B or Htr2A was applied into chondrocytes. Inhibition of Htr1B or Htr2A by each si-RNA blocked serotonin-induced expression of Cox2, iNOS, and IL-6 in chondrocytes. Tph1 overexpression in chondrocytes showed up-regulated catabolic and inflammatory factors. Etopic expression of Tph1 in mouse cartilage tissue caused OA cartilage destruction. Interestingly, serotonin can act as an intercellular signaling molecule via its receptors, also intracellularly via regulation of the activity of target protein through covalent coupling of serotonin to them by transglutaminase 2 (TGM2). This coupling, called serotonylation activates small GTPases such as RhoA may induce OA cartilage destruction via increased catabolic and inflammatory factors. To determine whether these effects were due to TGM2-induced serotonylation when increased serotonin in arthritis-like condition, we treated LDN, a TGM2 inhibitor, and Y-27632, a Rho kinase inhibitor and confirmed the reduction of inflammatory factors. Taken together, our findings indicate that serotonin may be newly synthesized in OA cartilage by cytokine-induced Tph1 and following chondrocyte-derived serotonin plays a pivotal role in the regulation of inflammatory osteoarthritis.

P1739/B879

Promotion of Osteoblast Differentiation and Matrix Mineralization in Response to Quercetin Hydrate in *Vitro*.

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Skeletal homeostasis throughout a lifetime is modulated on the cellular level, chiefly between the actions of bone forming osteoblasts and bone resorbing osteoclasts. The interaction between these two bone cells is a novel area of study in order to identify agents which could potentially have therapeutic applications to benefit populations who are afflicted with bone degenerative disorders or those who have suffered fractures. Our lab focuses on naturally derived or plant-based supplements. Quercetin hydrate (QH) is a non-citrus flavonoid that has been studied for its antioxidant qualities in models of cardiac dysfunction, cancers, and inflammatory diseases. Previous studies from our lab have implicated

QH as an agent which positively promoted osteoblast proliferation and early markers of differentiation. The objective of our current study was to evaluate the effects of QH on pre-osteoblastic cell lines MC3T3-E1 and MG-63 at physiologically relevant concentrations. There were no treatment-induced cytotoxic effects in response to QH treatment. Cultures treated with QH showed significant increase in calcium and phosphate deposition with QH. There was also an increase in matrix mineralization with QH compared to untreated control. This data combined with our previously reported finding suggest QH can be integral in the promotion of osteoblast cell differentiation, matrix organization and maturation in *vitro*. This gives us a potential avenue to further explore QH and possible therapeutic applications of supplementation for the benefit of promoting skeletal health.

P1740/B880

Electron Microscopy Findings in Spinal Arachnoid Mater Calcifications.

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A number of white depositions were found in a 65-year-old male cadaver during the opening of the spinal canal. The depositions were found under the spinal arachnoid mater from thoracic to lumbar level. Histological examination were proceed to make diagnosis. We think this might represent a case of Arachnoiditis Ossificans (AO) which is a term used to describe a partial ossification of spinal arachnoid mater. The process is said to be related to previous trauma or surgery which might lead to dural and arachnoid mater inflammations which eventually lead to ossification. We would like to present electron microscopy findings and our hypothesis for the formation of bony deposit that is somehow related to cell wound healing.

P1741/B881

Inhibition and Recovery of Human Dental Pulp Cells Biomineralization in Extracellular Matrix.

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Formation of tertiary dentin via differentiation and mineralization of dental pulp stem cells is essential to support the architecture of the pulp chamber and protect the tissue from bacterial invasion. Triethyleneglycol dimethacrylate (TEGDMA), a toxic monomer that leaches out from the adhesive restorations close to pulp, reaches the tissue to inhibits dentin formation. The inhibitory effect of the monomer on mineral deposition in *vivo* and capability of N-Acetyl Cysteine (NAC) to recover this process can be successfully investigated by developing and characterizing a biomineralization extracellular matrix (ECM) platform. In this study, we employ the ECM platform to demonstrate NAC's ability to resume growth of cells affected by TEGDMA. Cell differentiation and mineral deposition are characterized at different time points (14 and 21 days) using alkaline phosphatase (ALP) detection and alizarin red S (ARS) staining procedures. Mineral spread pattern can be detected on a 2.5D culture of cells seeded on a thin layer of ECM and analyzed by quantitative phase imaging (QPI). At TEGDMA concentrations of 0, 0.5, 1.5 and 2.5 mmol/L and with administered 10 mmol/L NAC, the hydroxyapatite content, percentage area covered, and spatial organization are validated by Raman microscopy, ARS staining and confocal imaging of cultures stained with the fluorescent Osteoimage™ reagent. In induced platforms, after 14 days, the percentage of the differentiating cells and expression of ALP increased more than seven-fold compared to the non-induced counterparts. After 21 days, the mineral covered

approximately 78% of the entire area/frame. At early and late stages, the focal accumulation of the mineral replaced by a spreading pattern revealed a gradient with an optical thickness peak and distance of 3-4 μm and 500 μm, respectively. With increased TEGDMA concentration, the percentage area covered decreased 3-,18- and 85-fold, respectively. The administration of NAC prevented the formation/deposition of the mineral in healthy cells and recovered the mineralization in cells affected by TEGDMA. The percentage area covered by the mineral in cultures exposed to 1.5 mmol/L TEGDMA increased 6-fold when treated with NAC. In samples exposed to 1.5 mmol/L TEGDMA and treated with NAC, the hydroxyapatite peak (detected by Raman spectroscopy) increased more than three times compared to the non-treated ones. Our study authenticates the ECM platform as an efficient tool for studying the impact of dental leachables on cell biomineralization and identifying drugs that efficiently restore tissue functionality.

06

Immune System

P1742/B882

Wine Your Way to Good Health: Anti-inflammatory Effects of Resveratrol.

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Wine your way to good health: anti-Inflammatory effects of resveratrol Graziella Greco and Joshua B. Slee Department of Natural Science, DeSales University, Center Valley, PA 18034 Abstract Resveratrol, an antioxidant found in red wine and grapes, is thought to possess anti-inflammatory properties in relation to cardiovascular disease, and may have beneficial effects on incisional wound healing and the body's response to implantable devices. Liquid-liquid extraction of resveratrol from red wine was performed, and used to study its anti-inflammatory effects in *vitro*. After culturing Bovine Aortic Endothelial cells (BAOECs) in resveratrol to ensure there was no adverse effect on normal cell growth and morphology, it was concluded that there was no effect due to resveratrol addition. An established way to model inflammation in BAOECs is by using Tumor Necrosis Factor-α (TNF-α), which induces a large accumulation of actin stress fibers. Our data suggest that resveratrol pretreatment reduces the amount of actin stress fiber accumulation, caused by TNF-α, thus exhibiting anti-inflammatory properties. A large amount of inflammation in the cardiovascular system is caused by a wound to the endothelial layer. A wound healing assay was conducted to determine the wound healing properties of resveratrol. Resveratrol was shown to dramatically aid in the wound healing process, compared to an untreated control. Furthermore, the immune response of the body to implantable devices was investigated using aTHP-1 cell adhesion assay to polyurethane, a common biomaterial used in medicine. When a foreign material is introduced into the body, an immune response is stimulated, and monocyte-derived macrophages stick to the biomaterial, hindering its function. THP-1 cells are a good in *vitro* model of the monocyte-derived macrophages found in the immune system. THP-1 cell attachment to the polyurethane was significantly reduced in the presence of resveratrol. These data indicate that resveratrol possesses promising anti-inflammatory qualities that may prove to be useful in the prevention of cardiovascular disease, improving wound healing, and decreasing biomaterial rejection.

P1743/B883

Inflammatory Profile of Chronic Recurrent Multifocal Osteomyelitis of Jaw.

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Chronic recurrent multifocal osteomyelitis (CRMO), also known as chronic non-bacterial osteomyelitis (CNO), is a rare inflammatory disorder that primarily affects children. We analyzed the postoperative exudate through immunoprecipitation high performance liquid chromatography (IP-HPLC) from the mandibular lesion of 12-year-old Korean girl, and evaluated its pathological wound healing process. In order to obtain the postoperative exudate, a decompressive drainage apparatus was applied in the site of CRMO operation. After the first operation, the postoperative exudate was collected in 6 hours, 1, 4, 5 days until the exudate drainage was stopped. An d also after the second operation, the postoperative exudate was collected in 1, 2, 3 days. After the first biopsy of CRMO lesion, inflammatory reaction was enhanced by upregulation of TNFα, IL-1, IL-6, IL-12, IL-28, CD20, CD44, LL-37, CD54, and CD56, while macrophage activating proteins, CD68, lysozyme, and IL-8 were decreased. Particularly, anti-inflammatory factors, TGF-β1 and IL-10 were markedly decreased, and wound healing-related angiogenesis and osteogenesis proteins were consistently down-regulated. After the second decortication, inflammatory reaction was stimulated by increases of IL-6, CD31, IL-10, IL-12, COX2, CD20, and CD40, and concurrent activation of macrophages/monocytes-related factors, IL-8, lysozyme, and CD68, together with upregulation of wound healing proteins, CD31, MMP-2, MMP-10, and RANKL during the postoperative 3 days. The present study showed the reduced expressions of angiogenesis and osteogenesis-related proteins after the first operation, but the angiogenesis and osteogenesis-related proteins were slightly increased after the second operation. Besides that, there was few evidence of adaptive inflammation caused by bacterial infection by consistent down-regulation of TNFα, CD3, IL-28, cathepsin C and G. Therefore, it was presumed that the surgical treatment is necessary to remove the inflammatory lesion of CRMO to reduce the pro-inflammatory cascade, and simultaneously to activate the innate antigen presenting cells (macrophages/monocytes). *This study was supported by grant no 03-2019-0043 from the SNUDH Research Fund

P1744/B884

Investigating the Role of Low-grade Inflammation in Osteoarthritis Progression.

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Osteoarthritis (OA) is a degenerative joint disease that is primarily characterized by cartilage destruction. Growing evidence indicates that both OA and metabolic syndrome (MetS) are low-grade inflammatory condition with elevation in systemic inflammatory mediators. However, the exact role of low-grade inflammation in OA pathogenesis has not been fully investigated. Here, we examined whether chronic low-grade inflammation affects experimental OA in mice. An d, we identified possible key factors responsible for the low-grade inflammation in OA pathogenesis. We established two experimental mouse models in this study. First, C57BL/6 male mice were fed either low-fat diet (10

kcal% from fat) or a high-fat diet (60 kcal% from fat) starting at 8 weeks of age and operated destabilization of the medial meniscus (DMM) at 12 weeks of age. Mice were sacrificed at 6 weeks after DMM operation and subjected to histological and biochemical analysis. Second, DMM-operated 12-week-old male mice were intraperitoneally injected with either phosphate-buffered saline or lipopolysaccharide (5 ng per kg body weight) twice weekly for 1 month. Mice were sacrificed at 8 weeks after DMM surgery and subjected to histological and biochemical analysis. Microarrays were used to analyze the transcriptome of chondrocytes stimulated by interleukin-1β (IL-1 β) treatment or hypoxia-inducible factor (HIF)-2α, or the zinc importer, ZIP8 overexpression. Our data shows chronic low-grade inflammation exhibited increased severity of OA caused by DMM surgery in mice. Our microarray analysis revealed that, from among the examined inflammation-related genes, Toll-like receptor (TLR) signaling components were differentially expressed in chondrocytes stimulated with IL-1β, Ad-HIF-2α or Ad-ZIP8 using microarray analysis. Overall, our findings demonstrate the possible role of TLR signaling components as the mediator of low-grade inflammation and OA which may help to establish promising therapeutics for the treatment of OA.

P1745/B885

Benzydamine Inhibits Osteoclast Differentiation and Bone Resorption Via Down-regulation of Interleukin-1β Expression.

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Bone diseases such as osteoporosis and periodontitis are induced by excessive osteoclastic activity, which is closely associated with inflammation. Benzydamine (BA) has been used as a cytokine-suppressive or non-steroidal anti-inflammatory drug that inhibits the production of pro-inflammatory cytokines or prostaglandins. However, its role in osteoclast differentiation and function remains unknown. Here, we explored the role of BA in regulating osteoclast differentiation and elucidated the underlying mechanism. BA inhibited osteoclast differentiation and strongly suppressed interleukin-1β (IL-1β) production. BA inhibited osteoclast formation and bone resorption when added to bone marrow-derived macrophages and differentiated osteoclasts, and the inhibitory effect was reversed by IL-1β treatment. The reporter assay and the inhibitor study of IL-1β transcription suggest that BA inhibits nuclear factor-κB and activator protein-1 by regulating IκB kinase, extracellular signal regulated kinase and p38, resulting in the down-regulation of IL-1β expression. BA also promoted osteoblast differentiation. Furthermore, BA protected lipopolysaccharide- and ovariectomy-induced bone loss in mice, suggesting therapeutic potential against inflammation-induced bone diseases and postmenopausal osteoporosis.

P1746/B886

Parkin Negatively Regulates Bone Resorbing Activity of Osteoclast.

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Dysfunction of Parkin (Park2) is linked to the progression of parkinsonism which contributes to a progressive systemic skeletal disease characterized by low bone mineral density. However, the role of Parkin in bone erosion has not been elucidated yet. In this study, we observed that Parkin expression was decreased in osteoclast (OC) progenitor cells, monocyte/macrophages, but markedly increased in

mature OC. Parkin overexpression in bone marrow-derived macrophages did not affect the differentiation of these cells. However, knockdown of Parkin using siRNA significantly induced bone resorbing capacity of mature OC, indicating that loss of Parkin promotes OC activity. These results suggest that Parkin negatively regulates osteoclastic bone resorption, implying the possible contribution of Parkin to the maintenance of bone turnover balance.

P1747/B887

Cellular and Secreted ApoE Protein Expression After Inflammation Is Glia Cell-type and *APOE*-Genotype Specific.

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Neuroinflammation is a common feature in neurodegenerative diseases, such as Alzheimer's disease. Inflammation in the brain is characterized by activated glial cells and increased levels of cytokines and chemokines. A protein with immunomodulatory properties is apolipoprotein E (apoE). ApoE is synthesized in the brain and modulates the inflammatory response of microglia and astrocytes. In humans, apoE exists in three isoforms, apoE2, apoE3 and apoE4. The apoE4 isoform increases an individual's risk for Alzheimer's disease; whereas the apoE2 isoform is considered neuroprotective. We hypothesized that E4 glial cells have a more pronounced pro-inflammatory response compared to E3 and E2. We took advantage of human *APOE* targeted replacement mice where the human *APOE2, E3* and *E4* genes have been introduced into *APOE* knockout mice. This allows the study of various apoE specific isoforms without the influence of mouse apoE. We isolated both astrocytes and microglia from these mice. Conditioned media and cells were collected and apoE was analyzed by western blot. Since apoE undergoes post-translational modifications, and these modifications can alter the way the protein migrates on a SDS-PAGE gel, we first determined the migration pattern of cellular and secreted apoE in glial cells. Our data showed that secreted apoE in astrocytes is characterized by two species, whereas secreted apoE in microglia by a single species. Cellular apoE in astrocytes is associated with one of these species, whereas microglial cellular apoE by both. Next we tested the effects of *APOE* genotype on the expression of apoE by glial cells following a pro-inflammatory stimuli. We used LPS and TNFα, two well-known pro-inflammatory molecules. E2, E3 and E4 glial cells were exposed to LPS (5-500ng/mL) or TNFα (3-30ng/mL) for 24 hrs in serum deprived conditioned media. E4 astrocytes released more apoE than E2 or E3 astrocytes, only at the highest dose of LPS (500ng/mL). In contrast, E2 and E3 microglia released more apoE than E4 microglia at moderate and high doses of LPS (50-500ng/mL). No changes in cellular apoE content was observed in either cell type. In contrast to LPS, TNFα reduced the secretion of apoE in E4 astrocytes. The rank order of reactivity was E4>E3=E2. Interestingly, TNFα stimulated the secretion of apoE in E4 microglia, without altering the secretion of apoE in E3 or E2 microglia. Together, our data suggest that activation of the innate immune response by LPS and TNFα results in opposing outcomes for secreted apoE in microglia and astrocytes.

P1748/B888

Prostaglandin $F_{2\alpha}$ Exaggerates LPS-induced Sepsis in Mice.

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Sepsis is the systemic inflammatory response to infection. It is known that the levels of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is increased in the plasma of the patients with sepsis. However, the functions of $PGF_{2\alpha}$ in the regulation of sepsis remains unclear. We investigated the role of $PGF_{2\alpha}$ in the progression of sepsis.

Sepsis was induced by intraperitoneal injection of 5 mg/kg LPS. In the treatment group, mice were intraperitoneally administrated with AL8810 (10 mg/kg) at 30 min before LPS administration. LPS administration promoted $PGF_{2\alpha}$ production in peritoneal lavage fluid (PLF). LPS administration enhanced the gene expression of proinflammatory cytokines such as IL-6, IL-1β, and TNF-α in lung and liver. In contrast, these gene expression was decreased by the administration of AL8810. It is known that neutrophil-derived IL-10 attenuates sepsis. At 6 hr after the LPS administration, the number of neutrophils and anti-inflammatory IL-10 levels in PLF were increased. In addition, inhibition of FP receptors further elevated neutrophil migration and IL-10 levels. Immunostaining analysis showed that IL-10 was detected in Gr-1-positive neutrophils in PLF. Then, administration of anti-IL-10 antibody increased LPS-induced gene expression of IL-6, IL-1β, and TNF-α in lung and liver. Furthermore, anti-IL-10 antibody administration enhanced AL8810-decreased expression of these genes. We concluded that inhibition of FP receptors attenuated the LPS-induced sepsis in mice. Administration of AL8810 promoted LPS-mediated IL-10-induced neutrophil migration and ameliorated the LPS-induced sepsis.

P1749/B889

The Decrease in Expression of Monocarboxylate Transporter-1 in Response to LPS Is a Secondary Event of the Inflammatory Response of Monocytes.

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The molecule Toll-like Receptor-4 (TLR4) recognizes Gram negative bacteria, specifically the lipopolysaccharide component (LPS) and initiates a signal transduction cascade leading to an inflammatory response. Recent studies by this laboratory suggest the cell adhesion glycoprotein Basigin (CD147) interacts with TLR4 via their transmembrane domains. The role of Basigin in mouse retina has been well-studied and includes an association with Monocarboxylate Transporter 1 (MCT1) for metabolic support of photoreceptor neurons. The role of Basigin in innate immune responses is less-well studied, but a recent study by this laboratory indicated that Basigin and MCT1 transcript expression is significantly reduced in response to LPS. The purpose of the present study was to assess the expression of Basigin and MCT1 protein expression in response to LPS. Expression of another metabolic transporter, the glucose transporter GLUT-1, was also assessed. It was hypothesized that Basigin and MCT1 protein expression would decrease in response to LPS and that GLUT-1 expression would increase to indicate a metabolic shift in response to the inflammatory stimulus. To test this, RAW 264.7 mouse monocytes were incubated in the presence of 1 μg/mL LPS in serum-free medium for 24 hours. Control cells received PBS. The cells were subjected to immunocytochemical analyses or were collected for protein isolation and subsequent ELISA analyses. Both methods indicated that the expression of Basigin and MCT1 proteins was significantly decreased in response to LPS treatment, when compared to the control cells. Conversely, the expression of GLUT-1 protein was similar between the control and LPS-treated cells. These findings do not support the hypothesis. Although it was initially proposed that monocytes undergo a shift of resources from general cellular maintenance to induction of an innate immune response through down-regulation of MCT1 expression, the lack of compensation by an increase in GLUT-1 expression does not support this. It is likely that MCT1 expression is influenced by Basigin expression, as it is in the neural retina, and the reduced expression of MCT1 reflects regulation of Basigin by the immune response in monocytes. Future studies will assess the mechanism through which Basigin and MCT1 are regulated by LPS and TLR4 during an inflammatory response.

P1750/B890

Interleukin-22 Induces Duffy Antigen Receptor for Chemokines through Stat5 in Monocytes.

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The atypical chemokine receptor 1 (ACKR1), also known as Duffy antigen receptor for chemokines (DARC) exhibits binding capacity for a wide variety of C-C and C-X-C chemokines that regulate inflammation. However, regulation of DARC expression has been largely unknown. Here, we found that Interleukin-22 (IL-22) regulates immune response by enhancing DARC expression in monocytes. STAT5 signal inhibitor abolished the IL-22-dependent increase in DARC as evidenced by immunoblotting and flow cytometry. The chromatin immunoprecipitation assay revealed that STAT5b directly binds to the DARC promoter. In addition, when we constructed DARC promoter constructs with a luciferase reporter containing wild type or mutated forms of the putative STAT5 binding site, we found that the predicted target sites, TTCCs is required for STAT5-dependent DARC expression. Our findings provide insights into the molecular mechanism by which IL-22 signal modulates STAT5-dependent DARC expression in monocytes.

P1751/B891

Macrophage Specificity: Identification of Polyethylene Glycol-resistant Macrophages on Stealth Imaging in Vitro Using Fluorescent Organosilica Nanoparticles.

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Macrophages have key functions in tissue homeostasis and inflammation. Macrophages are the special cells of differentiation of the mononuclear phagocyte system and show marked heterogeneity in response to environmental stimuli. The heterogeneity of macrophages was characterized by using morphology, surface marker, single cell sequence and so on. We are investigating macrophage heterogeneity to demonstrate macrophage specificity using our nanotechnology. We are developing novel technology of organosilica nanoparticles and performed in vitro imaging to evaluate the diversity and specificity of mouse macrophage uptake. Surface-functionalized organosilica nanoparticles with polyethylene glycol (PEG) were prepared by a one-step process, resulting in a brush-type PEG layer. A simultaneous dual-particle administration approach enabled us to evaluate the stealth function of nanoparticles with respect to single cells using time-lapse fluorescent microscopic imaging and flow cytometry analyses. Single-cell imaging and analysis revealed various patterns and kinetics of bare and PEGylated nanoparticle uptake. The PEGylated nanoparticles revealed a stealth function against most macrophages (PEG-sensitive macrophages); however, a stealth function against certain macrophages (PEG-insensitive macrophages) was not observed. We identified and characterized the PEG-resistant macrophages that could take up PEGylated nanoparticles at the same level as bare nanoparticles. These results indicated heterogeneity of macrophage uptake depending on surface structure of the particles. Our approach showed high possibility to characterize uptake diversity and specificity of macrophages. Reference: [1] M. Nakamura, S. Ozaki, M. Abe, T. Matsumoto and K. Ishimura, *J. Mater. Chem.,* **21** (2011) 4689. [2] M. Nakamura, A. Awaad, K. Hayashi, K. Ochiai and K. Ishimura, *Chem. Mater.* **24** (2012) 3772. [3] M. Nakamura, K. Miyamoto, K. Hayashi, A. Awaad, M. Ochiai and K. Ishimura, *Nanomedicine: NBM*, **9** (2013) 274. [4] M. Nakamura, K. Hayashi, M. Nakano, T. Kanadani, K. Miyamoto, T. Kori, K. Horikawa. *ACS nano*, **9** (2015) 1071. [5] M. Nakamura, K. Hayashi, H. Kubo, T. Kanadani, M. Harada, T. Yogo. *J*

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P1752/B892

Exosomal Mirna19a from Human Nasal Epithelial Cells Induce Pro-inflammatory Responses of Macrophages.

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Macrophages are phagocytes that play a critical role in the immune system. They are involved in both innate and adaptive immunity by either removing pathogens or antigen presentation. It has been shown that macrophages are polarized to subgroups with distinct functions, M1 and M2. M1 polarization is characterized by pro-inflammatory and anti-cancer functions, whereas M2 macrophages promote immune suppression and tumor growth. In this study, we investigate that exosomal miRNAs from human nasal epithelial cells play roles in airway mucosal microenvironment. Specifically, we demonstrated that the human nasal epithelial cells treated with urban dust modulate airway mucosal microenvironment through upregulation and release of miRNAs. The result shows that the human nasal epithelial cells treated with urban dust altered miRNA expression profile and released miRNAs promotes macrophage polarization. The study will further investigate exosomal miRNA mediated-communications between epithelium and macrophages in airway mucosal microenvironment.

P1753/B893

Effects of Statins on the Inflammatory Response of Macrophages Activation through the Tlr3 Signaling Pathway.

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Statins, HMG-CoA reductase inhibitors, are lipid-lowering medications. Recently, statins have pleiotropic effects such as antioxidant and plaque stability, which are cholesterol independent effects. However, statins have also been reported to exert immunosuppressive effects against viral and bacterial infections, Thus, statins cause so various unexpected problems. The aim of this study is to investigate the effects of statins such as simvastatin and pitavastatin, on poly(I:C)-activated macrophages. Macrophages were pre-treated with statins for 24 or 48 hrs, followed by treated with 10 μg/mL of poly(I:C) for 4 hrs. The expressions of the IFN-β, IP-10/CXCL10, RANTES/CCL5, IFIT, and TLR3 genes, which are induced by viral infection, were significantly reduced by treating with simvastatin or pitavastatin. Furthermore, these statins impaired the poly(I:C)-induced IRF-3 phosphorylation and the production of IFN-β. These suppressive effects of statins were effectively cleared by adding mevalonate or geranylgeranyl pyrophosphate, but not farnesyl pyrophosphate and cholesterol. These results suggest that simvastatin and pitavastatin have the inhibitory effect of IFN-β production on poly(I:C)-activated macrophages, and this effect may be due to the suppression of the TLR3 pathway. Furthermore, these suppressive effects of statins appear to be exerted via the Rho pathway.

P1754/B894

Phagocytosis Increases After Disruption of *Cis* Interactions between the Macrophage Checkpoint Receptor SIRPa & CD47.

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Checkpoint receptors on an immune cell generally allow a ligand on a 'self' cell to signal against immune activation, and in the case of macrophages, SIRPα signals against engulfment by binding CD47 expressed on all cells - including macrophages. Here, inhibiting *cis* interactions between SIRPα and CD47 on the same macrophage increases phagocytosis approximately the same as inhibiting *trans* interactions. Antibody blockade of CD47, as pursued in clinical trials against cancer, is applied separately to human-derived macrophages or to red blood cell (RBC) targets for phagocytosis, and both scenarios produce surprisingly similar increases in RBC eating. Knockdown of macrophage CD47 likewise increases engulfment of foreign cells and particles, decreases SIRPα's baseline inhibitory signaling, and linearly increases binding in *trans* of soluble CD47, consistent with mathematics of *cis-trans* competition. Soluble SIRPα binding to human-CD47 displayed on Chinese hamster ovary (CHO) cells is indeed suppressed by SIRPα co-display, with molecular simulations confirming SIRPα can bend over and bind CD47. Safety and efficacy profiles for CD47-SIRPα blockade can thus reflect disruption of *cis and trans* interactions.

P1755/B895

Ratio of Activating to Inhibitory Signaling Dominates Phagocytic Decision-Making.

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Cancer immunotherapies involving macrophages broadly aim to modulate their effector function in one of two ways, either by increasing phagocytic activating signals or decreasing inhibitory signals. To increase activating signals, therapeutic antibodies targeting cancer antigens are introduced to drive antibody-dependent phagocytosis through activating Fc receptors (FcRs) on the macrophage. To decrease inhibitory signals, blocking antibodies targeting macrophage inhibitory receptor SIRPα (or its ligand CD47) are introduced to enable the macrophage to bypass this inhibitory checkpoint, thereby removing the brakes on macrophage effector function. In each strategy, the goal is to tip the balance of activating vs. Inhibitory receptors present at the interface between the macrophage and target cell, either by promoting activating FcR engagement or by preventing inhibitory SIRPα engagement. However, little has been shown quantitatively about how macrophages integrate these competing receptor signals to ultimately determine phagocytic behavior. Here we demonstrate that macrophage phagocytic decisions are finely tuned to the ratio of activating ligand (antibody) to inhibitory ligand (CD47) present on the target cell. Using reconstituted cell-like target particles with defined surface proteins, we found that the same activation-inhibition ratio drives similar levels of phagocytosis across a broad range of absolute molecule densities. To test the dependence of this ratiometric decision-making on specific receptors, we examined a panel of synthetic activating and inhibitory receptors and found similar results, indicating this ratiometric paradigm applies broadly to both endogenous and artificial (e.g. CAR) immune receptors. Our results demonstrate that ratio—not an absolute threshold—dominates macrophage phagocytosis decisions. This understanding of how macrophages integrate contradictory signals may be useful when designing combination immunotherapies. Additionally, this study suggests that characterization of tumor surface composition could be critical for determining patient-specific immunotherapy regiments.

P1756/B896

Emergence and Systemic Redistribution of Tumor-resident Dendritic Cell Subsets.

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Dendritic cells (DC) are cellular sentinels that gather and distribute tissue-derived antigens to coordinate adaptive immunity, including anti-tumor responses. Recent studies demonstrated that DC subsets residing in the tumor microenvironment can drive infiltration and activation of cytotoxic T cells. However, the origin of these DC subsets from circulating vs. tissue-resident precursors is poorly defined. More broadly, scant data exists on the temporal dynamics of DC influx into and redistribution out of tumors despite their central role for anti-tumor immunity. These gaps in knowledge have stymied attempts to map the wide variety of DC behaviors in *situ* onto DC of different origins. We dissect the expansion and behaviors of tumor-resident vs migratory DC using lineage tracing and intravital imaging. We first asked whether DC are generated within the tumor microenvironment or are recruited from circulation. Over several days, we tracked lineage-marked tumor-resident or migratory DC within tumors and draining lymph nodes. Distinct populations emerged at both sites that were not mirrored by newly infiltrating DCs, suggesting the tumor microenvironment may profoundly dictate differentiation of DC subtypes in *situ*. We correlated these trends at the population level with behaviors of tumor-resident DC using intravital imaging over consecutive days. Quantitative image analysis of DC revealed cell morphological and cell motility shifts that corresponded with the generation of tumor-resident DC subsets. Finally, we monitored DC trafficking out of tumors into the periphery in real time and over consecutive days by coupling an in *vivo* cytometry platform with photolabeling to mark tumor-resident DC. We quantified and captured tumor-experienced cDC shed into the periphery on consecutive days from the same mouse, demonstrating the feasibility of this approach for future studies. These observations reveal the origins and behaviors of DC residing in tumors and prompt novel avenues to perturb DC subsets, thereby improving immunotherapies for cancer.

P1757/B897

Role of Advanced Glycation End Products in Neutrophil Extracellular Trap Formation.

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Soon after injury or microbial contamination, neutrophils exit the circulation and move to the affected area, where they generate reactive oxygen species (ROS) that damage and kill microorganisms and produce chromatin-based neutrophil extracellular traps (NETs) that trap bacteria and fungi and facilitate neutrophil-mediated microbial killing. While ROS and NETs are critical in preventing infection, they also damage nearby host cells, causing more inflammation and tissue damage. This positive feedback loop ends in a process called inflammation resolution, leading to decreased neutrophil recruitment to the area, reduced production of pro-inflammatory ROS and NETs, and increased neutrophil apoptosis. Previous studies have suggested that chronically elevated glucose levels in type II diabetes increase NET formation, which may contribute to the prolonged inflammation and reduced wound healing seen in patients with this disease. In this study, we are investigating whether the advanced glycation end products (AGEs) produced by prolonged hyperglycemia promote NET formation through the AGE receptor for advanced glycation products, RAGE. To do so, we induced NET formation in HL-60-derived neutrophil-like cells with phorbol myristate acetate (PMA), in the presence and absence of two types of AGEs, AGE-modified bovine serum albumin (AGE-BSA) or Nε-(1-Carboxymethyl)-L-lysine. We then

quantified NET formation by fluorimetry of treated cells stained with Sytox Green, which primarily stains extracellular DNA. Because histone H3 is citrullinated during NET formation, we also used fluorescence microscopy of treated cells stained with anti-citrullinated histone H3 antibody to identify cells undergoing NETosis. Our results demonstrate that both AGE-BSA and Nε-(1-Carboxymethyl)-L-lysine exacerbate NET induction in neutrophil-like HL-60 cells. We then examined the role of RAGE in this AGE-enhanced NET formation by treating HL-60 neutrophils with AGEs in the presence and absence of FPS-ZM1, a RAGE inhibitor. We found that the RAGE inhibitor prevented the AGE-enhanced production of NETs, implicating RAGE in AGE-enhanced NET production. These results suggest the intriguing possibility that AGE-mediated RAGE activation contributes to the excessive NET production seen in hyperglycemic type II diabetic patients.

P1758/B898

Gambogic Acid from *Garcinia Hanburyi* Inhibits the Production of Neutrophil Extracellular Traps in *Vitro*.

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The innate immune system responds rapidly to early signs of an infection or tissue damage by recruiting neutrophils to the affected area, where these inflammatory cells kill microbes through the production of reactive oxygen species (ROS) and neutrophil extracellular traps (NETs). Although these inflammatory responses are necessary to eliminate potential pathogens before they proliferate and establish an infection, ROS and NETs are non-specific mechanisms that can also damage host tissues. In chronic inflammation associated with various diseases, such as rheumatoid arthritis or Crohn's disease, prolonged or non-resolving inflammation can cause severe pain and permanent tissue damage. We are currently investigating the potential of gambogic acid, a compound derived from the Garcinia hanburyi plant used in Ayurvedic and traditional Thai medicines, to reduce inflammation and/or promote its resolution. Previous studies found that gambogic acid promotes apoptosis in multiple cell types, suggesting that this molecule might promote apoptosis in neutrophils and thereby inhibit their inflammatory functions. Because separate studies suggest that apoptosis interferes with NET formation, we investigated whether gambogic acid could reduce neutrophil survival and production of NETs. To determine the impact of gambogic acid on NET formation, we treated neutrophil-like HL-60 cells with gambogic acid in the presence and absence of phorbol myristate acetate (PMA), a known NET inducer, and used fluorimetry to quantify NET formation after staining cells with Sytox Green, which primarily detects extracellular DNA. We found that gambogic acid decreases PMA-induced NETs in these neutrophil-like HL-60 cells. We then used the colorimetric WST-8 cell survival assay and LDH release assay to quantify cell survival and necrosis, respectively. We found that gambogic acid reduces the survival of HL-60-derived neutrophil-like cells, as shown by their decreased metabolic activity and increased membrane permeability after treatment with gambogic acid. Furthermore, the inhibitory effect of gambogic acid on NET formation was reversed if cells were treated with Boc-D-FMK, a pan-caspase inhibitor, providing evidence that gambogic acid inhibits NET formation by promoting apoptosis. We are currently performing additional experiments to confirm that gambogic acid induces neutrophil apoptosis and also using more specific caspase inhibitors to better understand the mechanisms through which gambogic acid may promote apoptosis and reduce NET formation in these cells.

P1759/B899

Disruption of TFAM in T Lymphocytes Leads to Impaired Mtdna Copy Number Regulation, Altered CD8+ T Cell Effector Function and Metabolism.

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Mitochondria contain multiple copies of maternally-inherited DNA (mtDNA) that encodes for 13 proteins all important for energy production via the respiratory chain. Loss of mtDNA copy number or integrity is associated with mitochondrial depletion syndromes - clinically heterogenous disorders caused by mutations in genes important for mitochondrial maintenance. Mitochondrial transcription factor a (TFAM) is an essential protein that binds mtDNA regulating packaging, replication, mitochondrial transcription, and mtDNA copy number and mitochondrial biogenesis. Total disruption of TFAM by gene targeting in mice results in embryonic lethality, while heterozygous mice exhibit reduced mtDNA copy number and respiratory chain deficiency in cardiac tissue. Although TFAM has been shown to also play a role in neurodegeneration, its contribution to mitochondrial maintenance in other tissues with high energetic demands remains poorly understood. To mount effective immune responses, T cells migrate between tissues, clonally expand, and secrete effector molecules; highly energetic processes known to be dependent on changes in metabolism. To better understand the role of mtDNA copy number regulation in T cell function, we disrupted TFAM expression in T cells using a loxP-flanked *Tfam* allele in combination with a Cre-recombinase transgene under the control of the CD4 promoter. TFAM-deficient T cells undergo normal thymic development and exhibit expected total numbers in the periphery. While T cell receptor-mediated activation of naïve CD8+ T cells results in reduced proliferative capacity, stimulated CD8+ T cells display increased expression of activation markers and enhanced cytotoxic function, on a per cell basis. Although mtDNA content is significantly reduced in TFAM-deficient T cells, MitoTracker staining suggests increased mitochondrial mass. Studies are currently underway investigating the bioenergetics and expanded immune responses in TFAM-deficient T cells. By understanding how loss of TFAM alters T cell metabolism, we expect to contribute to our knowledge of the link between mtDNA copy number regulation and immune cell function. This work has the potential to aid in the development of improved strategies for evaluating and treating immune dysfunction in patients with mitochondrial disease associated with mtDNA depletion.

P1760/B900

A Splice Acceptor Variant Associated with Multiple Sclerosis Affects Hla-dra Conformation and Cellular Localization.

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The strongest genome-wide association signal in multiple sclerosis (MS), an autoimmune disease of the central nervous system, maps within the major histocompatibility complex (MHC). This locus encodes a large group of proteins governing both adaptive and innate immune responses. Among them, MHC class II proteins form alpha/beta heterodimers on the membrane of professional antigen-presenting cells (APCs), where they display pathogen-derived exogenous antigens to CD4+ T lymphocytes. In a recent fine-mapping effort on a cohort of 1,427 MS cases and 1,203 controls with African American ancestry, we have identified a splice acceptor variant (rs8084) in the alpha-chain encoding *HLA-DRA* gene, which is in complete linkage disequilibrium with the major MS risk allele *HLA-DRB1*15:01*. This variant mediates the transcription of an alternative version of the alpha-chain lacking 25 amino acids in its extracellular

portion. Although this shorter isoform is still able to form stable heterodimers with the corresponding beta-chain, it is not clear how changes in its conformation may affect its function in the context of antigen presentation and MS susceptibility. In this regard, we have performed detailed molecular dynamics (MD) simulations suggesting the stability of the short DRA is significantly lowered due to the absence of a disulfide bond between two beta-sheets in its alpha2 Ig-like domain. Nonetheless, the dimerization domain remains intact in agreement with the experimental data. Also, the relative orientation of these two domains is changed, leading to a reduction in the radius of gyration of the protein. Such extensive structural rearrangement dramatically affects the maturation process of short DRA, which remains entrapped into the endoplasmic reticulum as confirmed by immunofluorescence experiments in cells expressing the short isoform along with the beta chain. However, short DRA is detected on the cell surface upon co-expression with the standard MHC receptor and co-immunoprecipitation assays clearly indicate this isoform binds the alpha/beta heterodimer. Due to its more compact conformation, protein-protein docking predicts that short DRA can potentially bind the native HLA-DRB1-DRA heterodimer and affect antigen presentation. Further experiments will be required to elucidate whether this isoform can function as a possible neo-antigen for autoreactive CD4+ T cells and assess its putative role in central nervous system (CNS) autoimmunity and MS pathogenesis.

P1761/B901

Elucidation the Role of ADAR1 in the Innate Immune Response.

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Adenosine deaminases acting on dsRNA (ADARs) are essential for normal embryonic development and are essential in preventing the innate immune being activated by endogenous dsRNA. ADARs deaminate adenosine to inosine by hydrolytic deamination, known as A-to-I editing. Our group was the first to demonstrate that this editing event in endogenous dsRNA prevents the interferon (IFN) signalling cascades from dsRNA sensors in the cytoplasm: RIG-I and MDA5. In accordance, mice that are null for *Adar1* die at embryonal stage E12.5 with heightened levels of type-I IFN and widespread apoptosis. In humans, mutations in *ADAR1* cause the autoimmune disorder Aicardi Goutières syndrome (AGS). Most of the AGS mutations reduce the editing activity of the enzyme, except *ADAR1 D1113H*. This mutation is in the deaminase domain and it is possible that it can lead to perturbation in protein-protein interactions. To address this, we investigated the ADAR1 interactome under different conditions. We have prepared a tetracycline-inducible HE239T stable cell line, expressing both the long and short isoform of ADAR1. These proteins were tagged with Strep-tag or BirA at either N- or C-terminus. To further elucidate biological functions of ADAR1p150, cells were treated with type I IFN. In addition, we have induced immune response in HeLa cell line and performed co-IP of ADAR1 to determine the protein complexes formed at the endogenous level. Taken together, we currently have a comprehensive data set of ADAR1 protein complexes with or without induction of immune response; both with IFN and HMW Poly I:C. Our data are consistent between all sets and in agreement with all published interacting proteins of ADARs. In addition to the different protein complexes found to interact with ADAR1 upon immune response, we have found that tags at the different terminus strongly influences protein stability and interactions. This work was supported from European Regional Development Fund-Project ,,A-C-G-T“ (No. CZ.02.1.01/0.0/0.0/16_026/0008448).

P1762/B902

Regulation of Osteoclast Differentiation and Function by Ew6.

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Excessive osteoclastic activity causes many bone diseases, such as osteoporosis and rheumatoid arthritis, of which control thus has significant clinical implications. Here we found that EW6 significantly inhibited the receptor activator of nuclear factor-kB ligand (RANKL)-induced osteoclast differentiation and its function. It attenuated the activation of NF-kB leading to the decreased NFATc1, a key transcription factor of osteoclast differentiation, as well as c-fos. EW6 exhibited abnormal actin ring formation via facilitating proteasomal degradation of integrin signaling molecules, such as p130cas, src and G proteins, leads to inhibition of bone resorption activity in differentiated osteoclasts. These results suggest that EW6 inhibits RANKL-induced osteoclastogenesis via regulation of the activation of NF-kB and also the osteoclast maturation by modulating stability of proteins which control morphology of osteoclast and function. In keeping with the results, EW6 inhibited bone loss in an inflammation- and Ovariectomy- induced bone erosion model. Thus, EW6 is promising drug candidate for bone related disorders.

New Techniques in Single Molecule and Super-resolution Microscopy

P1763/B1

Development of the Green Photo-switchable Fluorescent Protein with Fixation Resistance, a Variant of Eos Fluorescent Protein.

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Super-resolution microscopy has overcome the diffraction limit of visualization of fluorescent molecules. One of the super-resolution microscopies, photoactivated localization microscopy (PALM), reconstructs images by the position of each fluorescent molecule, that is determined by the Gaussian fitting of the stochastically observed single molecule fluorescence. Such observation can be enabled by the blinking of the fluorescent proteins or molecules. To obtain larger number of protein positions for image reconstruction, larger numbers of images were required, and thus, sample fixation is required for large number of image collection during longer duration. However, most fluorescent proteins for PALM imaging lose their fluorescence and blinking ability after fixation. Here, we made novel green reversibly-photo-switchable fluorescent proteins with fixation resistance, named frSkylans, by combining the amino acid sequences of mEos4b, which is a photo-converting protein from green to red with fixation resistance, and those of Skylans, which are green photo-switchable proteins without fixation resistance, since they all are derivatives of EosFP. frSkylans showed higher fixation resistance than Skylans and retained photo-switching ability in cells. Their fixation resistance enabled larger number of signal acquisition and higher resolution of the reconstructed images by PALM observation, as shown by frSkylan-tagged vimentin or clathrin light chain expressed in fixed U2OS cells, showing that frSkylans can be used as fluorescence tag for PALM observation with green fluorescence.

P1764/B2

Integrated Super-resolution Imaging Toolbox for Diagnosis of Motile Ciliopathies.

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Lung clearance of pathogens and particulates relies on motile cilia. Impaired cilia motility can lead to reduction in lung function, lung transplant or death. Over 40 proteins regulating cilia motility are linked to primary ciliary dyskinesia (PCD), a heterogeneous recessive genetic lung disease. Accurate PCD molecular diagnosis is essential for identifying therapeutic targets, initiating therapies that can stabilize lung function and reducing the economic and social impact caused by this disease. To date, PCD diagnosis has mainly relied on non-quantitative methods either with limited sensitivity or requiring a-priori knowledge of the genes involved. Here, we developed a super-resolution microscopy toolbox aimed at addressing current limitations in PCD diagnosis: 1) to increase sensitivity, we built a localization map of PCD proteins by 3D-structured illumination microscopy (3D-SIM) and implemented quantitative image analysis to detect protein mis-localization; 2) to detect nanometer-scale structural defects in PCD patients' cells, we analyzed axonemal structure by stochastic optical reconstruction microscopy (STORM); 3) to detect motility defects caused by yet to be discovered genes, we developed a quantitative super-resolution method to detect motile cilia un-coordination by rotational polarity. Altogether, we show that super-resolution methods are powerful tools for improving molecular diagnosis of motile ciliopathies.

P1765/B3

Whole Cell 3D Single Molecule Localization Microscopy Using Single-Objective Selective Plane Illumination Microscopy (soSPIM).

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Assessing protein organization and dynamics in their native cellular context provides invaluable insights into their activities and functions. The development of super-resolution microscopy approaches, allowing imaging proteins of interest with spatial resolution down to the nanometer scale, has been a great step forward in this direction. Amongst these techniques, Single Molecule Localization Microscopy (SMLM) enables localizing and tracking biomolecules in their cellular environment with the highest spatial resolution. However, this resolution is critically dependent on strong background rejection, limiting the penetration depth of standard SMLM implementation to the first micron above the coverslips using TIRF or HiLo. Conventional 3D imaging methods like confocal and Selective Plane Illumination Microscopy (SPIM) lack the sensitivity to efficiently localize single molecule events. To overcome those limitations, we developed the soSPIM technique, which uses a single high numerical aperture objective in combination with microfabricated devices. This architecture benefits from both a high collection efficiency and an optimal optical sectioning in depth, allowing to perform SMLM tens of microns above the coverslip[1]. Here we will present the capabilities and requirements for soSPIM to probe the 3D nanoscale organization of proteins in depth at the single-molecule level. This involves the use of adaptive optics for aberration-free astigmatism-based 3D localization as well as a computerized method for real-time mechanical drift correction. We will illustrate this work by showing the quantitative 3D nanoscale organization of several membrane proteins of entire T-Cells. **References:** R. Galland, G. Grenci, A. Aravind, V. Viasnoff, V. Studer, JB. Sibarita, “3D high- and super-resolution imaging using single-objective SPIM”, Nature Methods, 2015, **12**(7): p. 641-44

P1766/B4

Quantifying IRES-mediated Viral Translation Dynamics with Single Molecule Resolution in Living Cells.

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Viruses use Internal Ribosome Entry Site (IRES) sequences to efficiently hijack host ribosomes away from the canonical 5’ cap and to a viral RNA. Although viral infection mechanisms have been studied extensively for years, the translation dynamics of the non-canonical mechanisms that viruses employ remain unexplored in living cells at the single-molecule level. To visualize host ribosome hijacking with single RNA resolution, a bicistronic construct with an IRES of interest between two reporter probes was developed. Nascent chain tracking (NCT) of this construct was employed to track and monitor single molecules of translating mRNA through time. Within single cells, mRNA were observed to exist in three different populations: Cap-only, IRES-only, and both Cap and IRES translation. Counting the number of translation sites per cell revealed that Cap Only translation occurred more frequently than IRES Only and Cap+IRES translation. However, interestingly, Cap+IRES translation occurred more frequently than expected if Cap and IRES initiation were two independent events, suggesting cross-talk between Cap and IRES translation within single translation sites. To further dissect the differences in translation efficiency,

we measured the ribosomal occupancy of translating mRNA. Intensity measurements of mRNA translating in either a Cap or IRES manner were compared revealing that Cap open reading frames (ORFs) have twice as many occupying ribosomes as IRES ORFs. Run-off assays revealed this difference was not due to elongation, but rather due to initiation, with Cap initiation occurring nearly twice as fast as IRES initiation. Viruses that harbor IRES elements often utilize a cell's stress response to shift the translational landscape to favor viral IRES-mediated over canonical Cap-dependent translation. After addition of a cell stressor to our system, we observed a significant initiation shift toward IRES Only translation. Interestingly, we also observed an increase in the number of Cap+IRES translation events after stress, further indicating a cross-talk between Cap and IRES translation within single translation sites. Altogether, our system allows us to fairly and accurately measure the efficiency of Cap- versus IRES-dependent translation with spatial resolution on the tens of nanometers scale and temporal resolution on the seconds timescale. Given the ubiquity of viral translation, we anticipate our biosensor will find broad application to better understand viral biogenesis in different cellular conditions.

P1767/B5

Applying Genetic Code Expansion and Bioorthogonal Labeling to Quantitative High-resolution Live Cell Imaging.

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Genetic code expansion (GCE)-based bioorthogonal labeling is an emerging approach for fluorescence labeling of proteins in live cells. In this approach, proteins are directly labelled with fluorescent dyes at 1:1 ratio. The superb photophysical properties of fluorescent dyes together with the minimal size of the tag (an order of magnitude smaller than GFP) hold great potential for improving the performance of current methods for quantitative imaging and super resolution microscopy. However, in spite of its potential, this approach is not widely used in live cell imaging applications. Here, we set out to expand the use of this approach and test its performance in quantitative single molecule applications. To this aim, we: 1) optimized conditions for intracellular and extracellular labeling of proteins in live mammalian cells; 2) engineered a 14-residues long N-terminal peptide tag for straightforward bioorthogonal labeling of essentially any cellular protein and constructed a library of GCE-tagged organelle markers; and 3) calibrated conditions and performed Single Particle Tracking (SPT) and live-Single Molecule Localization Microscopy (SMLM) of bioorthogonally labeled proteins in mammalian cells. Conclusively, our results show that GCE and bioorthogonal chemistry is a suitable, flexible approach for protein labeling in quantitative high-resolution live cell microscopy that outperforms current live cell labeling approaches. This opens up new opportunities for quantitative analysis of cellular processes in the cellular milieu with improved spatiotemporal accuracy.

P1768/B6

Identifying Patterns of Protein Organisation in 3d Super-resolution Datasets.

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Super-resolution microscopy techniques can now precisely locate specific molecules within cells and cellular structures, to within ~10 nm. However, proteins within macromolecular complexes are organized at length scales that are still very difficult to access. Single-particle averaging techniques

adopted from electron microscopy show some promise, but are of limited use in many fluorescently-labelled samples. As well as limits on precision for the location of each detected molecule, low labelling density of a sample, and heterogeneity between and internal to macromolecular complexes, pose significant problems. We have developed a novel approach to determine protein organization with these complexes that overcomes these problems, by aggregating the relative positions between localisations to reveal common organizational features. We applied this new analysis technique to super-resolution data acquired for several different molecular organizations. We first used 3D STORM data for a nuclear pore (Nup107) protein, which has known rotational symmetry, and provides a good test for our approach. We successfully extracted accurate organizational information (symmetry and distances) from this data, demonstrating the strength of our technique. Next, we tested 3D STORM data for cardiomyocyte Z-disks. A major component protein of the Z-disc, α-actinin-2 (ACTN2) was labelled using a novel small non-antibody binding protein (Affimer, Tiede et al., 2017;6:e24903), conjugated to Alexa 647. Z-disks are crucial for muscle structure and contractility, and contain a repeating complex, which has a dense, inhomogeneous, ~20-nm tetragonal lattice structure. We successfully uncovered this known repeating pattern of ACTN2 across the Z-disc (~20nm repeat) along the long axis of the muscle fibre. A similar analysis of 3D PALM data using mEos-tagged ACTN2 in Z-discs was also successful despite low labelling and detection efficiency of the labels, and an average localization precision comparable with the lattice length scales. 3D PALM data for Eos-tagged myopalladin and LIM-nebulette, or LASP2) showed novel organizational features, suggesting a new model for the localization of LASP2. Using these, and other examples, we have demonstrated that this tool can be used to find pattern information from samples with unknown structures, and expect this analysis to be applied to study regularity and symmetry in many other complexes.

P1769/B7

High Speed Single Molecule Dynamics in the Endoplasmic Reticulum.

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The endoplasmic reticulum (ER) is an expansive, membrane-enclosed organelle that plays crucial roles in numerous cellular processes. Besides its clearly defined roles as the major site of cellular translation, the master regulator of calcium homeostasis, and origin of the secretory pathway; it also maintains elaborate and highly dynamic contacts with essentially all the other subcellular organelles. Despite this clear role as a central regulator of many important processes in cell biology, the way in which the many functions of the ER are spatially regulated has remained enigmatic. Diffraction-limited imaging experiments have failed to identify clear separation between components of ER biology, suggesting that functional microdomains within the membrane or lumen are either too small or too transient to be detected by conventional imaging techniques. Here, we correlate high speed single molecule trajectories of proteins in the endoplasmic reticulum to simultaneously collected imaging data of the context in which the molecules are moving. We show this approach works well with structured illumination microscopy, hyperspectral imaging, and even simple diffraction-limited imaging using a standard commercial widefield scope. By analyzing the trajectories of ER-localized proteins in the context of the ER itself, we show that functional microdomains do frequently exist in the ER, however they appear to be highly regulated in both space and time. For some classes of proteins, the existence of these domains is highly dependent on the underlying membrane structure, while others are transiently recruited to sites of interaction with specific cytoplasmic factors, often contact sites with other

organelles or large protein complexes. Collectively, this data suggests correlating single molecule trajectories into cellular context may provide a valuable tool for connecting biological process regulation across diverse spatial scales.

P1770/B8
Versatile Tools Towards Real-time Single-molecule Biology.
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Biological processes performed by proteins interacting with and processing DNA and RNA are key to cell metabolism and life. Detailed insights into these processes provide essential information for understanding the molecular basis of life and the pathological conditions that develop when such processes go awry. The next scientific breakthrough consists in the actual, direct, real-time observations and measurements of the individual mechanisms involved, in order to validate and complete the current biological models. Single-molecule technologies offer an exciting opportunity to meet these challenges and to study protein function and activity in real-time and at the single-molecule level. Here, we present our efforts for further enabling discoveries in the field of biology and biophysics using both the combination of optical tweezers with single-molecule fluorescence microscopy. We show the latest applications in protein conformation, folding, and unfolding; protein droplet fusion during liquid-liquid phase transitions; DNA-protein interactions and genome modifications; effects of mechanical stress on DNA/RNA structure; motility of cytoskeletal molecular motors; cell receptor force activation; as well as various other intracellular dynamic processes.

P1771/B9
Investigation of Lentiviruses and Their Glycoproteins Using Real-time 3D Tracking.
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Lentiviruses live double lives either as therapeutic vehicles or a threat, and both Jekyll and Hyde behaviours are mediated through studded envelope glycoproteins (Env). The generation of lentiviral vectors (LVs) through replacement of cognate Envs with versatile, more promiscuous, classes of Envs with broader cellular targets has become a main gene-editing delivery system. Therefore, our ability to understand Env incorporation into mature viruses will not only give us improved strategies to defend ourselves but also our ability to harness them as beneficial gene therapy tools. While the number, organization, and distribution of Env protrusions on the HIV-1 surface is engrained in literature from structural and modelling experiments, less is known about Env incorporation into lentiviral vectors. Here we focus on one of the most commonly used lentiviral vectors, whereby the G protein of vesicular stomatitis virus (VSV-G) decorates the surface of the viral membrane and try to understand what controls the number of these novel Envs on custom designed lentiviral vectors. Mature lentiviruses live fast and die young, thus, to study their actions requires a microscope that is capable of keeping up. In this work, we track, at unprecedented timescales (10 μsec to minutes), single rapidly diffusing and fluorescent virus-like-particles (VLPs) in solution using a 3D Dynamic Photon Localization Tracking (3D-DyPLoT) microscope. This enables both the size and Env number to be determined for each individual virus. Active feedback tracking by 3D-DyPLoT is achieved using a dynamically scanning laser pattern and converting photon arrival times into 3D positional information. In real-time, the diffusing particles' position is continually updated and held within the center of the continually scanning laser spot using a piezoelectric stage, relaying high spatiotemporal diffusion and intensity information. We find that the

number of VSV-G Envs incorporated into integration-competent lentiviral vectors is low, on average there were 12 ± 2 Envs per VLP, but the vast majority of VLPs were skewed to a very low number, one or two, of incorporated Envs. 3D-DyPLoT can adapt to different diffusive regimes enabling us to track VLPs on their journey to the cell surface and initial membrane-binding events taking advantage of the full potential of this system to characterize Env-receptor interactions. With LVs being more commonly used in clinical applications, regardless of their payload, successful targeting of LVs to nominated cells is largely dependent on the interaction of Envs with endogenous membrane receptors. Ultimately, results here can help to design effective LVs with fit-for-task envelope glycoproteins.

P1772/B10

Simultaneous Multi-Color 3D Whole Cell Super-Resolution Imaging and Particle Tracking Using Engineered Point Spread Functions.

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Widefield fluorescence optical microscopes are the workhorse of laboratories in numerous fields including biology, medicine and pharmacology, providing invaluable visual information and informative quantitative data. The function of bio-molecules is often inferred by comparing the behavior of different intercellular functions by labeling them with different molecules. It is therefore valuable to localize and track multiple colors simultaneously in 3D. However, currently available microscopes are often limited in their imaging depth, resolution, and ability to image different fluorophores simultaneously. Current solutions rely on sequential imaging or an additional camera and do not provide the required flexibility to work with the recently available large sensor formats (25 mm diagonal) and the ubiquitous legacy format (11.6 mm diagonal). Furthermore, none of the existing solutions take advantage of the increased depth, speed, and 3D resolution enhancements provided by engineered PSFs[1]-[3]. Here, we present two unique solutions that overcome these limitations and enable simultaneous 3D extended depth multi-color imaging of biological samples. We address the limitations of existing solutions in two ways. Our first solution is based on multi-color phase masks (MC-PM) [4], which uniquely identify two or more probes on the same camera sensor area by encoding the spectral information, and thus 3D position, into a distinct PSF for each color. An example of this is shown in Fig 1 where the MC-PM encodes DH-PSFs at perpendicular orientations for two selected wavelengths. Our second solution is a universal modular subsystem - SPINDLE-2C, which can be installed between most scientific microscopes and cameras. Along with a library of engineered PSFs including the MC-PMs[4], Double-Helix[1], Tetrapod[3], the SPINDLE-2C can be used with dichroic mirrors or polarizing/non-polarizing beam splitters. It is easily adjusted to work with a wide range of camera formats with no loss of resolution across the full field of view. The module features interchangeable mounts enabling users to optimize the PSF for their experiments, while allowing easy switching between single color, dual color, and bypass modes.The unique combination of multimodal image and engineered PSFs has been used to extend the imaging capabilities of microscopes for nanometer-scale single-molecule imaging[1], [2], light sheet[5], 3D particle tracking [3], [4], in the study of cancer[6], immunology, neuroscience[7], and more.

P1773/B11

Live Simultaneous Multi-receptor Tracking in Neurons with 5-D Single Molecule Localization Microscopy.

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Single particle tracking (SPT) techniques such as sptPALM, uPAINT, and quantum dot tracking have given unprecedented insight into molecular dynamics in living cells. They allow monitoring the behavior and molecular interaction of individual proteins at millisecond temporal resolution and high spatial resolution (<30 nm) by fitting the point spread function (PSF) of individual emitters and tracking their position over time. While these SPT methods have been extended to study the temporal dynamics and co-organization of multiple proteins, conventional experimental setups used to perform multicolor imaging are typically limited to two simultaneous wavelengths. Increasing the number of colors requires additional filters for specific fluorescent tags and is usually performed at the expense of spatial or temporal resolution and/or field of view. This limits the minimum diffusion coefficient that can be measured and reduces the statistics that can be gathered from a single experiment, thereby degrading the ability to differentiate between molecular diffusion regimes like immobilization and confined diffusion. Moreover, simultaneous multi-receptor tracking could also reveal specific interactions between different protein populations, which could previously only be inferred from the behavior of a single population without any knowledge of the presumed partner behavior. By employing a dual-objective imaging configuration compatible with routine live cell imaging, we will present a single molecule tracking technique that allows for simultaneous 3D single particle tracking of multiple distinct species without compromising spatio-temporal resolution. A dispersive element introduced into the second optical path induces a spectrally-dependent displacement, which is used to separate numerous fluorescent species of single emitters based on their emission spectra. A proof of concept of the spectral separation abilities of the system will be shown via simultaneous 3D DNA-PAINT of fixed samples, where the acquisition time is significantly reduced compared to conventional sequential multicolor imaging. Lastly, we will demonstrate how the technique can be applied to track multiple receptors in live neuron cultures, and we will discuss possibilities of how advanced data analysis techniques can fully exploit the 5-dimensional data (x,y,z,t,λ) to extend the capabilities of conventional single particle tracking, such as the investigation of protein-protein interactions.

P1774/B12

Live-cell Imaging and an alysis of the Plasma Membrane Dynamics during Clathrin-mediated Endocytosis by High-speed Atomic Force Microscopy.

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Clathrin-mediated endocytosis (CME) is a process through which cells incorporate extracellular materials and membrane proteins into the inside of cells. Such a process is accompanied by a series of morphological changes of the plasma membrane, which is mediated by a successive assembly of many protein components. Although the protein assembly has been elucidated by fluorescence microscopy,

dynamic changes in the plasma membrane morphology during CME have not been fully clarified in living cells. We have previously developed a combined system of a fast-scanning atomic force microscope and a confocal microscope and performed simultaneous imaging of morphological changes of the plasma membrane and protein dynamics in living cells. Through this observation, we have revealed that actin polymerization at a clathrin-coated pit (CCP) provides a major force to drive membrane protrusion and push vesicle downward for irreversible closing of CCPs in an Arp2/3 complex-dependent manner (Yoshida et al., PLoS Biology, 2018). In this study, with the use of the combined system, the dynamic changes of plasma membrane morphology during CME in the presence of extracellular stimuli were studied. When serum-starved Cos-1 or A431 cells that stably express Venus-fused clathrin-light chain a were stimulated by EGF, a division and frequent appearance of CCP were observed. This resulted in the clustering of CCPs in the limited area of the membrane with a diameter of 800-1600 nm. This phenomenon was conserved among cell lines because it was observed in both Cos-1 and A431 cells. The clustering of CCP was suppressed by the treatment with EGF receptor inhibitors. Furthermore, upon the closure of CCPs in EGF-stimulated cells, it was frequently observed that membrane protrusion collectively covered and capped the clustered CCPs. Taken together, these results indicate that there exists a domain on the plasma membrane, where CME frequently emerges, and the formation of such the membrane domain is maintained by EGF receptor activity. In conclusion, contrary to the previous assumption that clathrin assembly is induced by ligand binding to the receptors, cells appear to have a “pre-opened gate” for coming extracellular materials to be incorporated.

P1775/B13

Mutation in the *HTT Gene* Critically Affects the Cell Motility and Alter Cytoskeleton Organization in Cultured Dermal Fibroblast of Patients with Huntington's Disease.

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Huntington's disease (HD) is a severe and currently uncurable hereditary autosomal dominant neurodegenerative disease, effected by the progressive death of the medium spiny neurons of the striatum, leading to death after 8-15 years since the manifestation. The cause of HD development is an expansion of CAG repeats in the *gene* of huntingtin protein (HTT). Despite the fact that this mutation has the greatest impact on neural cells, it is shown that HTT is expressed in all cells and tissues of mammals. The function of HTT in humans is not clear, however, it is revealed that HTT interacts with proteins involved in (1) transcription, (2) signal transduction in the cell and (3) intracellular transport. We investigated morpho-functional disorders occurring at the cellular level and affecting subcellular cytoskeletal structures (microtubules and actin components). The primary fibroblasts obtained from the skin biopsy of HD patients with different length of CAG repeats in the *HTT gene* (42, 44 and 76) were used as the object of the study. Fibroblasts from skin biopsies of healthy donors corresponding to patients by sex and age have been applied as control. Physiological tests showed that fibroblasts of HD patients have different characteristics of spreading and movement compared to fibroblasts from healthy donors. They have a shorter stage of spreading, are capable to polarize before complete cell spreading and start the movement before polarization stage is fully completed. Patient’s fibroblasts do not form a pronounced leading edge and tail at the stage of polarization, their lamella forms numerous thin outgrowths on the leading edge during the process of attachment and spreading on the substrate. During the experimental wound healing fibroblasts of HD patients moved faster than control cells, but

their movement was more chaotic - they often changed direction of movement compared to normal cells. Confocal microscopy and SIM showed that the structure of the microtubule network does not change critically in the cytoplasm of HD fibroblasts. However, the architecture of cytoplasmic actin network changes and the mutual arrangement of γ - and β-actin structures is disturbed. Herewith the total amount of γ - and β -actin proteins does not differ from that in fibroblasts of healthy donors. In patient's cells the process of formation of primary cilia is violated: the number of cilia was lower, and their average length was less than in fibroblasts of healthy donors. Thus, our results had shown for the first time that in Huntington's disease morphological disorders caused by mutations in the *HTT gene* affect the cell cytoskeleton structures, which leads to changes in cell spreading and motility. Supported by RSF (#19-15-00425) and MSU Development Program PNR 5.13.

P1776/B14

***In Vitro* Actin Cytoskeleton Organization during Endothelial Monolayer Integrity Formation and Regulation Revealed by Super-Resolution Microscopy.**

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Endothelial cells have a flattened shape and, tightly adjacent to each other, form a layer of cells lining the inner surface of blood vessels. The endothelium plays a barrier role and regulates vascular tone and vascular wall permeability. However, the endothelial monolayer is sensitive to the effects of various physical and chemical stimuli, both in physiological and pathological states. The barrier function efficiency is dependent on the interaction of cell cytoskeleton structures and adhesive (cell-cell and cell-substrate contacts) components. All three types of cytoskeletal structures (microtubules, actin and intermediate filaments) operate coordinately through various linkers and mediators, contributing to the strengthening or weakening of the endothelial barrier. Initially, barrier function and dysfunction researches were focused on the actin system of the endothelial cells. Actin structures are an essential component for cell contraction. In endothelial cells, both in *vivo* and in *vitro*, two actin isoforms are present: non-muscle β- and γ-cytoplasmic actin. In this study, double immunofluorescent labeling and super-resolution microscopy (Structural Illumination Microscopy, SIM), as well as correlation analysis, were used to study the relative position of cytoskeleton components consisting of different actin isoforms during endothelial monolayer integrity formation and regulation. The results of the study demonstrate significant differences in actin structures intracellular localization - β-actin is found mainly in stress fibers and ring bundles, and the extensive actin network of the cell cytoplasm consists of γ-actin. In the area of lamellipodia, near to complete colocalization of cytoplasmic β- and γ-actin structures is often observed, but simultaneously, in the same endotheliocyte, colocalization is not observed in the zone of stable cell edges and in cell central regions surrounding the cell nucleus. Relative localization of β- and γ-actin components changes during the formation of endothelial cell monolayer. Similar changes are observed during endothelial barrier dysfunction development resulting of experimental microtubule- depolymerizing treatments. The reported study was supported by Russian Foundation for Basic Research (project number 18-29-09082) and Moscow State University Development Program (PNR 5.13).

P1777/B15

High Speed, High Throughput Super-resolution Imaging.

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Single-molecule switching (SMS) nanoscopy techniques like STORM/(F)PALM circumvent the diffraction limit by sacrificing temporal resolution. SMS typically requires several minutes to reconstruct a single super-resolution image, severely limiting its throughput. High-speed SMS has been achieved using high laser power and sCMOS cameras, however, conventional analysis pipelines cannot handle the resulting 70 TB/day data volume. We have overcome this by developing a platform for high-throughput SMS, enabling us to perform automated super-resolution imaging of ~10,000 3D fields of view a day. Our integrated system comprises a biplanar-astigmatism microscope specifically designed to produce high volumes of 3D and two-color SMS data as well as a computer cluster for real-time analysis. It can record SMS images of whole nuclei (25 x 25 x 4 um) in 8 s, and can image autonomously for at least 30 hrs. The computer cluster runs PYthon Microscopy Environment (PYME) software, which we have developed to include a microscopy-specific compression algorithm and distributed storage framework to receive the full 800 MB/s bandwidth of sCMOS cameras in real-time. We additionally use the computer cluster to run our now fully GPU-accelerated sCMOS-specific localization algorithms to localize 39,000 emitters/s, which is real-time at our 800 Hz imaging rate for up to 49 emitters/frame. The distributed analysis backend in PYME additionally supports batch-processing post-localization analysis recipes over thousands of super-resolution images or point-clouds. Our platform converts SMS imaging from a qualitative or small-scale quantitative tool into a valid imaging technique for large-scale quantitative hypothesis testing. After preliminary high-throughput demonstrations characterizing nuclear phenotypes, we are now leveraging our advances to study the organization of the interphase nucleus, specifically studying the size and positioning of lamin-associated domains with respect to the nuclear envelope.

P1778/B16

Single Particle Motions of Axonal Proteins Captured by a Confocal Laser Scanning Microscopy.

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Fluorescence correlation spectroscopy is an established single-molecule based method for a variety of measurements in the field of protein science, such as estimating the concentration and diffusion coefficient of proteins, which enables to calculate a dissociation constant (Kd) of proteins, and so on. There are several difficulties in applying these methods, and its application in cell biology is still limited and a challenging work. To overcome these problems, we developed a new FCS methods based on the confocal laser scanning microscopy (CLSM) equipped with high speed scanner combined with an image processing technology. CLSM is also used in image correlation spectroscopy (ICS), but this is usually applied for molecules on plasma membrane. Even if soluble proteins in cells are measured, the accuracy of estimation is limited caused by fluctuation of cells or organelle. Our methods succeeded in accurate and multi-point calculation of auto- and cross-correlation functions in living cells. In addition, the cross correlation between pixels enabled us to extract one directional flow of proteins which have only been achieved by FCS equipped with multi-detector systems. Some neuronal axons extend over several meters long. In axons, motor proteins, such as kinesin and dynein, enable to transport synaptic vesicles and other soluble proteins. We modified our methods to optimize a balance between scan speeds and

S/N ratio to visualize single particles. At last, we could capture trajectories of single particles diffusing in axon. We applied these methods to several axonal proteins, and discussed quantitative results of them.

P1779/B17

Optimized Expansion Microscopy of Chromatin.

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Imaging biological structures at high resolution is critical for understanding their functions. Due to the diffraction limit in light microscopy, the maximum achievable resolutions in the lateral and axial dimensions are approximately 250 nm and 500 nm respectively, which are lower than the size of protein complexes in the cell. Expansion microscopy (ExM) is a super-resolution method based on physical expansion of a biological sample such as a monolayer of cells or a tissue slice embedded in a hydrogel. After expansion, the specimen can be observed by a wide field florescent microscope as well as super-resolution microscope to further improve the spatial resolution making it an economic tool for super resolution imaging. However, optimization is required to apply to chromatin-bound structures, such as the kinetochore. Although ExM shows potential to be used for the analysis of sub-cellular protein complexes, detailed characterization of chromatin expansion in three dimensions is lacking. To this end, we optimized two types of ExM, using either Methylenebisacrylamide (MBAA) or Dimethylacrylamide (DMAA) monomers. Three of major problems faced while imaging an ExM sample are photobleaching, sample drift, and anisotropic expansion. We achieved stable, high-fluorescence signal using an optimized mounting media. We developed a sample mounting chamber to constrain the sample with minimal drift in X,Y and Z dimensions and allow long-term imaging. Before applying the method to chromatin, we measured the isotropy of expansion. The MBAA gel expands 4 fold in all three dimensions. The DMAA gel expands to 10 fold in the lateral dimensions but only to 5 fold in the axial dimension. To assess whether expansion of chromatin is similar to the gel, we applied expansion to mitotic and interphase chromatin in HeLa cells. Both chromatin size and volume isotopically expand 3- and 6-fold for MBAA and DMAA, indicating that chromatin expansion is restricted (compared to gel expansion). To further improve uniformity in chromatin expansion, we investigated and optimized the use of proteinase K and micrococcal nuclease. We have further applied optimized ExM methods in MCF10a-organoid 3D cultures and found that the chromatin expands to 4 and 8 fold with MBAA and DMAA, respectively. We would like to share our optimized MBAA and DMAA ExM methods and discuss the potential to apply analysis of chromatin or chromatin-bound structures in the cell.

P1780/B18

Single-molecule Subcellular an alysis of Rna Distribution in Three Dimensions Using Open Source Tools.

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Imaging is a foundational component of cell biological research, as it allows investigators to interrogate relationships between cellular components and reveals unique aspects of cellular function. Quantification of imaging data discriminates between different biological states, reduces observer bias, and facilitates research rigor and reproducibility. Our laboratory investigates how localized RNA regulates the centrosome, a crucial organelle that coordinates cell division and cellular organization. To do this, we combine single-molecule RNA fluorescence in *situ* hybridization (smFISH) with

immunofluorescence imaging in the early *Drosophila* embryo. In these embryos, the centrosome is a small target within much larger cells, which makes precise and accurate analysis of RNA distribution challenging. While commercial software exists for spatial analysis of immunofluorescence images, licensing is often cost prohibitive and single-molecule RNA analysis is not supported. Several investigators have shared freely available software for spatial analysis of subcellular localization and/or quantification of smFISH data, but no open-source solutions exist. Here we present the methodology for an open-source pipeline written in Python to quantify the spatial distribution of smFISH data relative to subcellular structures of interest. We applied this pipeline to smFISH data for several putative centrosomal RNAs (cmRNAs) and detected subtle enrichments at centrosomes relative to a control RNA. Further, we developed and applied a batch processing mode of this pipeline, which enabled analysis of more than 50 images for each RNA type across nine stages of early *Drosophila* development, including both interphase and mitotic embryos. This batch analysis demonstrated that cmRNAs are dynamically regulated during the cell cycle and across developmental stages. We also found that multiple cmRNAs are packaged into RNA granules containing at least four transcripts that are enriched at the centrosome. Our pipeline can easily be generalized to any structure that can be segmented, including images of cells, tissues, and objects where spatial distribution is relevant to analysis. We conclude that open-source image analysis pipelines provide opportunities for trainees to gain useful programming skills and enable new biological discoveries without costly software licensing.

P1781/B19

Expanding the Limits of 3D Fluorescence Microscopy.

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Since the pioneering work of the Ed Boyden lab (Chen et al., 2015), a new family of sample preparation techniques termed expansion microscopy (ExM) has been rapidly developing. These techniques harness hydrogel-tissue chemistry—previously used for optical clearing—to physically enlarge samples, allowing super-resolution imaging with conventional microscopes. In ExM, samples are embedded in a swellable polyelectrolyte gel, and then undergo digestion or denaturation treatments which drastically reduce the strength of the biological material. Since the gel is densely crosslinked within the sample, when the gel swells, parts of the original sample are pulled away from each other in a uniform manner. This swelling is maximal in pure water due to the repulsion between the negatively charged groups in the gel. The typical linear expansion rate is ~4x, but up to 10x expansion in a single step has been reported. As with most complicated protocols, ExM is not a “one size fits all” approach. Due to the large variability of properties such as size, shape, and mechanical toughness in biological specimens, the protocol must be tailored for each type of sample. Consequently, multiple labs have developed and validated different protocols, even for the same sample types. Because each biological structure requires different considerations for preservation, the expansion microscopy protocol must be optimized and validated for every new set of biological structures. The goal of our laboratory is to facilitate the adoption of new techniques, such as ExM, by researchers at the National Cancer Institute in Frederick, MD who need to image a variety of samples, from viral particles, to cell organelles, to animal models, to tumors. Here, we report the impact of gel composition and gelation conditions, such as temperature and exposure to oxygen, on the performance of expansion microscopy, and present examples of our optimization toward simple and robust protocols.

New Techniques in Genomics and Proteomics

P1782/B20

Optical Pooled Screens.

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Pooled genetic screens have been critical for the systematic identification of genes underlying cellular processes, but have thus far been limited to phenotypes defined by cellular enrichment or comparatively low-throughput single-cell molecular profiling. We have developed a method to make pooled libraries compatible with the rich set of spatially and temporally resolved phenotypes accessible to high-content microscopy by using targeted in situ sequencing to demultiplex genetic perturbations. We applied this technology to screen 952 genes for involvement in NF-κB signaling by imaging p65 nuclear translocation and relaxation, recovering most canonical pathway members and identifying novel candidate regulators of IL-1β/TNFα-stimulated immune responses. We are currently piloting applications with a range of optical assays and cell models and expect that pooled optical screens will have broad utility in identifying genetic components, analyzing genetic circuits, and interrogating disease variants.

P1783/B21

Clonal an alysis, Genetic Screening and Expression Profiling at a Single-cell Level.

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Novel methods for profiling clonal composition and phenotypes of primary and metastatic lesions at the single-cell level are required for understanding tumorigenesis mechanisms and development of anti-cancer drugs with unique mechanisms of action. In order to facilitate these studies, we developed a panel of lentiviral barcoded libraries to label and monitor the cancer cells in time-course experiments in *in vitro and* mouse xenograft models. The barcodes are transcribed from lentiviral constructs and could be detected by NGS in a single-cell RNA expression profiling assay to identify the sub-population of descendant cells derived from a single barcoded progenitor cell. Furthermore, cell barcodes were incorporated in conjunction with genetic effector libraries, such as sgRNA libraries, to identify clonal phenotypic changes induced by specific genetic disruptions in progeny cells derived from the single progenitor cell. However, while barcoding cells provides an effective way to group cells based on clonal origin in heterogeneous cell populations, expression profiling at a single-cell level remains challenging. Data will be presented showing how genetic screening and targeted RNA expression profiling of human cells combined with cell barcoding could significantly improve phenotyping of distinct cell populations.

P1784/B22

Simple and Robust Tool for Discovery of Desired Gene Products Using a Genome Wide Full Length Cdna Transfection Array.

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Gene expression array technology has revolutionized the scientific community by making available expression profile of thousands of genes in a single experiment between normal and tumor tissues,

treated and untreated cell cultures, and across the developmental stages of an organism or tissue. However, it also has major limitation in that the expression profile of genes do not inform us about their function, causal or effect, or stimulatory or inhibitory with respect to disease or physiology, but only the abundance or lack thereof in the experimental sample. To overcome this challenge, to rapidly uncover the functions of genes and to identify gene products with desired properties, we have developed a revolutionary platform whereby Genome wide Full length cDNA (GFC) clones are transfected into cells and screened for a desired function. The desired function may include probing for a clone's effect on any of the following but not limited to such as modulation of transcription factors, signaling pathways, metabolic pathways, gene regulation, and glucose homeostasis. The GFC transcription array platform compared to others like the gene silencing method provides an alternate screening advantage with gain of function of genes rather than the inverse, and makes possible study of lethal genes that cannot be silenced. To establish this proof of concept we screened for transcription factor, NFAT (nuclear factor of activated T cells) or its activators among many other clones by measuring its specific ability to bind to its consensus sequence or response element (RE) to activate transcription of target genes. We co-transfected hundreds of full length human cDNA clones each in a separate well of a 96-well plate with the NFAT-RE luciferase reporter plasmid into the HEK293 cells. Two days post transfection the plate was analyzed for luciferase reporter activity. We found that all the NFAT transcription factor variants including NFATC1, NFATC2, NFATC3, and NFATC4 that we tested markedly stimulated luciferase activity by 9-30 folds compared to control (Luciferase reporter alone) and that this observation was reproduced in duplicate set of plate as well. Therefore, these findings establish the proof of concept of our GFC-transcription array platform. In conclusion, GFC transfection array platform presents as an excellent tool for discovering gene products with desired properties from a pool of unknown genes.

P1785/B23

Spatiotemporal Dissection of Single-Cell Proteome Plasticity in Human Cells.

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The Human Protein Atlas (HPA) project revealed that 17% of the human proteome displays significant expression variability between single cells within a seemingly homogeneous cell population. Understanding the cause and functional consequences of cell-to-cell variation remains an outstanding question in cell biology. Here, we present the first spatiotemporal dissection of cell cycle-independent proteome heterogeneity in HEK293T cells, with single cell resolution. We applied a high throughput CRISPR/Cas9-mediated approach for endogenous fluorescent protein tagging to generate a library of cell lines for the systematic study of phenotypic plasticity. Using a convolutional neural network for cell segmentation, we show that a wide variety of proteins, including metabolic enzymes, transcriptional regulators and proteasome subunits, exhibit cell-to-cell variation in their abundance or their spatial localization. We combine this spatial proteomics approach with mass-spectrometry and time-lapse confocal microscopy for in-depth characterization of the dynamics of protein expression variability. We identify proteins with time-dependent oscillatory expression, as well as proteins whose expression is influenced by the cell's microenvironment. Taken together, our results establish a framework for understanding the causes and functions of cellular heterogeneity in mammalian cells and tissues in health and disease.

P1786/B24

Distinguishing the Practical Applications of BioID and TurboID.

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Proximity-dependent biotin identification (BioID) has become a widely-used method for identifying protein-protein interactions (PPIs) within live cells and in several animal models. To accelerate the 15h-18h labeling period required for robust biotinylation, directed evolution of the BioID ligase was used to create TurboID and miniTurbo biotin ligases that can elicit robust biotinylation after a 10 min incubation with excess biotin. The potential applications for TurboID should allow for monitoring changes in PPIs that occur within minutes or a few hours following drug treatment or some other experimental variable. However, there is reported concern about biotinylation in the absence of biotin in some applications and possible toxicity in animal models. To determine the practical applications of TurboID and ascertain its practical strengths and weaknesses compared to BioID (and vice versa), we analyzed cell lines stably expressing BioID, TurboID, or miniTurbo constructs using several methods including immunoblot, immunofluorescence, and BioID pulldown proteomics. Our studies revealed considerable TurboID and miniTurbo fusion-protein instability, persistent biotinylation in the presence of basal levels of biotin, and a wider labeling radius compared to BioID in most standard applications. However, TurboID use in the lumen of the endoplasmic reticulum allowed for some improved degree of inducibility and overall improved biotinylation as compared to BioID. These studies should help inform investigators seeking to use BioID based methods as to the most appropriate ligase for their experimental needs.

P1787/B25

Proteome-wide an alysis of Cytoplasmic Meso-scale Organization.

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Key organization principles for the eukaryotic cytoplasm are the formation of stable protein complexes, membrane-bound organelles, and membrane-less condensates that utilize liquid-liquid phase separation (LLPS). Despite the importance of LLPS in cytoplasmic organization for many biological processes, it is still unclear what fraction of proteins organize themselves through this mechanism. Here, we assay the organization of proteins into meso-scale assemblies on a proteome-wide scale and reveal the underlying organization principles with molecular resolution. We filtered undiluted cytoplasm from frog eggs through porous membranes under different pressures. We quantified the relative permeation of each protein with multiplexed proteomics. Protein complexes are retained by small pores regardless of applied pressure. In contrast, liquid assemblies are retained by small pores at low pressure but squeeze through the same pores under high pressure. We find that well-known LLPS proteins showed the predicted differential behavior and can be clearly separated from stable protein complexes. We identified hundreds of proteins with similar differential characteristics suggesting that LLPS plays a wide and still mostly undiscovered role in the self-organization of the cytoplasm. This new methods to assay the meso-scale organization of cytoplasm suggests that many condensate organelles still remain to be discovered.

P1788/B26

AirID: a Novel Proximity Biotinylation Enzyme for an alysis of Protein-protein Interaction.

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Proximity biotinylation based on *Escherichia coli* BirA enzyme is thought as a key technology for identification of proteins interacted with a target protein in cell or organism. Firstly, BioID (proximity-dependent biotin identification) was reported, and its main improvement was a single mutation of R118G in BirA. BioID can be used just by expressing BioID fusion protein and adding biotin. Because a BioID-fusion protein biotinylates proximal proteins, it can comprehensively identify interacting proteins by precipitation with streptavidin beads and mass spectrometry. BioID can easily analyze interactome in mild condition. However, to biotinylate interacting proteins, BioID takes long time (>16 hours) and requires high concentration of biotin. Secondly BioID was improved by R118S and several mutations using yeast-surface display, and then TurboID was made. TurboID has extremely high activity and can biotinylate proteins in just 10 min. However, TurboID causes non-specific biotinylation and cell toxicity. Although BioID and TurboID are excellent enzymes, further improvements of BirA enzyme are important to enhance the convenience of the proximity biotinylation in cells. Here we have developed a novel BirA enzyme designed by an algorithm of ancestral enzyme reconstruction using the meta genome data classified by original method. Using this algorithm, five BirA sequences were created, and then these proteins were synthesized by the wheat cell-free system. RG or RS mutation of three proteins indicated the activity of proximity biotinylation like BioID or TurboID. We found a designed protein, named AirID (**A**ncestral B**ir**A for proximity-dependent biotin **id**entification), which showed high activity and specificity of proximity biotinylation. AirID-p53 or AirID-IκBα showed in *vitro* and in cell biotinylation of MDM2 or RelA, respectively. AirID-CRBN showed pomalidomide-dependent biotinylation of IKZF1 and SALL4 in *vitro*. Also, AirID-IκBα biotinylated endogenous RelA protein known as interactor of IκBα, and AirID-CRBN did endogenous CUL4 and RBX1 in $CRL4^{CRBN}$ complex. Mass spectrometry analysis also showed biotinylation of previously known interacting proteins. These results indicated that AirID is a novel and useful enzyme for analysis of protein-protein interaction.

P1789/B27

Membrane Protein Inventory of Human Pheochromocytoma and Paraganglioma Using a Multipronged "Pitchfork" Strategy.

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Pheochromocytoma and paraganglioma (PHEO/PGL) are rare neuroendocrine tumors. Prognosis of patients with malignant PHEO/PGL is poor, and specific molecular targets for new therapies are therefore painfully needed. Integral membrane proteins (IMPs) expressed by tumors represent optimal potential targets, due to their specific functions and localization. However, the amphipathic nature of IMPs, lack of trypsin cleavage sites in the hydrophobic segments and relatively low expression levels hinder proteomic analysis of IMPs. In order to maximize the membrane proteome coverage, we recently developed a multi-pronged “Pitchfork strategy” (1). The strategy employs four methods, each selectively targeting different features of IMPs: N-glycosylated peptides are isolated by two N-glycopeptide capture

methods (SPEG, glyco-FASP), hydrophobic transmembrane segments are targeted by the hPTC method using CNBr cleavage and hydrophilic non-glycosylated peptides are accessed by a standard detergent-trypsin method. We applied the four-pronged “Pitchfork strategy” to the membrane proteome profiling of human pheochromocytoma and paraganglioma (PHEO & PGL) samples. The individual ,,Pitchfork” methods are complementary and provide access to all cellular compartments. On average, we identified 900-1300 IMPs in each tumor sample or healthy adrenal medulla. It represents nearly 2600 unique human IMPs identified in all PHEO & PGL or control samples analyzed to date (n=22). Among the identified proteins, we observe several IMPs found only in the tumor tissue and absent in healthy adrenal medulla. Such candidate proteins are currently studied in detail as potential drug targets or disease markers. The Pitchfork strategy enables significantly deeper access into the realm of membrane proteome compared to conventional proteomic methods as documented by high number of identified IMPs and also by finding of numerous “missing proteins”, i.e. proteins with no previous evidence of existence on protein level. 1) Vit O, et al. A three-pronged "Pitchfork" strategy enables an extensive description of the human membrane proteome and the identification of missing proteins. J Proteomics. 2019 Jul 30;204:103411. doi:10.1016/j.jprot.2019.103411.

P1790/B28

Comparative an alysis of Postmortem Change in Bovine Skeletal Muscle Using Metabolomic Approach.

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This study aims to identify the metabolites in skeletal muscle from livestock using a metabolomic approach. Using gas chromatography-mass spectrometry (GC/MS), many metabolites were reproducibly detected in skeletal muscle, and distinct differences between Japanese black cattle and the crossbreed cattle were detected. Comparison of metabolites between skeletal muscle tissue (longissimus thoracis and semimembranosus) detected many metabolites in semimembranosus. Moreover, many amino acid and nucleic acid increased with aging after slaughter. Furthermore, an increase in free fatty acids was observed with aging. As a result of the multivariate analysis of these data, it was possible to determine the aging status of cattle by GC/MS analysis. These results suggest that metabolomics by GC/MS analysis can be used to evaluate beef quality.

New Techniques in Cell Biology 3: Tools

P1791/B29

Icy2.0: the Newly Redesigned Software for Bioimage an alysis.

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We present Icy2.0 (http://icy.bioimageanalysis.org), which is a major evolution of Icy, our previous free and open software for bioimage analysis. This release has three important features: 1) the ability to handle very large images (> 4Gb); 2) the possibility to process long sequences through an optimized streaming procedure with adaptive caching; and 3) a totally redesigned web site for improved interactions within the Icy community. Icy2.0 has retained and consolidated the major features that made its success in the bioimaging community, namely to make it available to the bioimaging community an image analysis software suite that encompasses the large variety of biological applications (microscopy, particle tracking, HCS/HTS, digital pathology, animal behavior,⋯) and users

(biologist, bioimage analyst, physicist, developer) and to give access to advanced image analysis methods and solutions. Since its launching, it has been the constant philosophy of the Icy tem to promote sharing of source code and know-how and to facilitate the use of quantitative approaches and open new scientific perspectives in terms of exhaustiveness, reproducibility and robustness of the analysis of bioimaging data sets. For those reasons, Icy2.0 has a fully redesigned web-site, that includes new communication channels between the end-users and the developers, new tutorial material and improved maintenance cycles. Icy2.0 now provides more than 400 dedicated plugins covering a large variety of state-of-the-art image analysis methods ranging from active contours models to Machine Learning through statistical spatial analysis, which empowers users with the most recent and adapted quantitative image analysis and visualization tools. Protocols, which are a graphical front-end that enable software development without any programming knowledge, have also been improved and now include the possibility to develop sophisticated image processing pipelines in a more readable and interactive manner. Icy2.0 has a large community of developers and users (4000+ regular users, 700+ students trained, 1500+ visits per month) that are using it for distributing their algorithms and plugins. For its long term sustainability and development, the Icy2.0 project benefits from the institutional support of the French national Infrastructure FranceBioImaging and of Institut Pasteur. Ongoing work aims at improving the interoperability and convergence of Icy2.0 with other open and free image analysis packages as well as facilitating the integration and use of AI packages. The Icy2.0 team is composed of Stéphane Dallongeville, Jean-Yves Tinevez, Daniel Fernandez Obando, Fabrice de Chaumont, Vannary Meas-Yedid, Thibault Lagache, Elodie Brient-Litzler, Robin Chalumeau and Piernicola Spinicelli.

P1792/B30

Application of Probe Electrospray Ionization to the an alysis of Blood and Neoplastic Diseases Combined with Machine Learning.

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Mass spectrometry (MS) is a powerful analytical tool enabling us to identify the molecules by *m/z*.In recent decade, it is one of the indispensable methods for biomedical sciences due to flourish of omics. For example, proteomic analysis uses MS to annotate the molecules in organelles, cells and tissues. However, there are several limitations inherent to this technique, such as destruction of cells and tissues, necessity of laborious pretreatments and ion suppression effect depending on the ionization methods. Probe electrospray ionization (PESI) is a derivative of ESI and overcomes some of these disadvantages. It does not require the multistep pretreatments of samples, and directly analyzes the living things with least intervention. PESI employs a very fine acupuncture needle serving both for a sample picker as well as for an ion emitter. Only several hundreds femto liter (fL) of sample allow us to obtain mass spectra sufficient for further analysis. Taking the advantage of very fine needle, we can apply it to the living animals to monitor the metabolic processes of internal organ such as liver. Moreover, this is applicable to discriminate the neoplastic region from the surrounding tumor-free regions. Speaking of MS analysis, another important issue to be reconsidered is the manner of data handling and processing. In conventional way of reductionism view, some significant or outstanding spectral peaks are somewhat arbitrarily selected for subsequent analyses. In our method, we make the most of whole spectral data without selecting specific peaks for annotation by any prejudice. Technically, mass spectra ranging from 10 to 2,000 are fed into the database to construct a reference for

judgment. For example, we collected around 200 tissue specimens from liver cancer patients and almost the same number of tumor-free region. After obtaining the mass spectra from these specimens, we built a database that contains combination of data specific to liver cancer and tumor-free liver tissue. Going through this database, classifier such as support vector machine (SVM) will give us the answer without annotating the molecules that underscore the molecular mechanisms of diseases. To date we achieved over 90% of concordance rate when compared with pathology. Other applications to the basic biological sciences will be presented on site.

P1793/B31

An Enzymatic Assay to Measure Adherence to Pre-Exposure Prophylaxis and Antiretroviral Therapy.

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Background. Antiretroviral therapy (ART) can extend the length and quality of life of people living with HIV and prevent HIV infection when given as pre-exposure prophylaxis (PrEP). However, sufficient medication adherence (≥ 4 doses/week) is required to prevent treatment failure and emergence of drug-resistant infections. Ongoing behavioral research aims to improve ART and PrEP adherence and drives the development of a growing number of strategies to measure ART and PrEP adherence. Drug-level measurement provides objective information about adherence patterns and is predictive of PrEP and ART healthcare outcomes. Tenofovir disoproxil fumarate (TDF) is used in 58% of ART and 100% of PrEP regimens. After ingestion, TDF is metabolized into tenofovir (TFV) and phosphorylated into tenofovir diphosphate (TFV-DP) - the active form of the drug. TFV-DP has a long half-life (17 days) making it an effective target for measuring adherence. The gold standard for TFV-DP measurement is mass spectrometry (MS), which is too capital-intensive, heavily-instrumented, and expensive for routine use in HIV endemic areas. Here we present a rapid enzymatic assay to measure TFV-DP. **Assay Principle.** TFV-DP competitively inhibits reverse transcriptase (RT) enzyme that synthesizes HIV cDNA. At *low* TFV-DP concentrations, RT is unlikely to incorporate TFV-DP into the cDNA chain and forms *long* cDNA strands that bind to many intercalating dye molecules and provide a *high* fluorescence signal, and vice-versa. **Experimental.** Master mixes consisted of RT, TFV-DP, a 200 nt DNA template, and dNTP in aqueous buffer and were incubated at 37°C for 30 min. PicoGreen intercalating dye was added and fluorescence was measured in a microplate reader. Data was analyzed using a 4-parameter logistic regression to calculate the 50% inhibition concentration (IC50). To simulate clinical samples, TFV-DP was spiked into whole blood diluted 1:15 by volume. **Results.** RT inhibition assays in buffer had the characteristic sigmoidal shape of enzyme inhibition assays with quantitative regions that could be moved lower or higher than the TFV-DP clinical range by adjusting dNTP concentration. We diluted blood in water to lyse red blood cells and minimize non-specific inhibition from the blood matrix. The assay distinguished TFV-DP spiked in whole blood at low adherence (1.7 doses/week) from medium (3.4 doses/week) ($p=0.038$) and high adherence (6.7 doses/week) ($p = 0.010$). **Conclusion.** We developed a rapid enzymatic assay to measure TFV-DP at clinically-relevant concentrations in whole blood. Ongoing work is focused on validating the assay with clinical samples, benchmarking against MS measurements, and integrating the assay into a near-patient format.

P1794/B32

A High-throughput Microfluidic Platform for Vasculature-ovary Carcinoma Tumor Explant Cultures.

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Predicting clinical response to anticancer drugs remains a major challenge in cancer therapy research. The current treatment of patients with ovarian cancer involves surgery and platinum-based chemotherapy, which still result in recurrence on advanced stages of the disease. During tumor progression, cancerous cells continuously accumulate mutations giving rise to a heterogeneous population of cells. Additionally, spatial distribution of stromal, endothelial and immune cells in the microenvironment affects tumor progression, cell morphology, and ultimately drug response. In order to predict clinical outcome of chemotherapy in *vitro*, tumor heterogeneity and microenvironment constituents must be conserved. Current models of tumor biology and microenvironment consist in xenografts of human tumors implanted in immunodeficient mice. These models allow studying systemic treatment response, but its application is limited to small developmental studies. Here, we present a high throughput in *vitro* 'grafting' platform where we co-culture blood vessels with tumor explants. Each unit in this platform is composed of two parallel microfluidic channels and a central chamber. Two endothelial tubules are generated in the microfluidic channels and cultured in presence of a gradient of angiogenic factors (S1P, VEGF, bFGF and PMA) added to the central chamber of the culture unit. An giogenic tubules form vascular beds within 3-5 days, after which tumor explants are loaded to the central chamber on top of the vascular beds. The model shown in this study consists of ovarian serous papillary adenocarcinoma collected after xenograft growth in immunodeficient mice. This ovarian cancer explant was previously characterized as resistant to Paclitaxel. In this study, we observe how the vascular bed remodels in the presence of the explant and closely interacts with ovarian tumor tissue. Vessel perfusion and stabilization of vascular bed was monitored by real time imaging of 150 kDa FITC-Dextran. Cultures were evaluated by assessment of morphology and presence of endothelial and tumor cell biomarkers. Moreover, co-culture response to Sorafenib (anti-angiogenic), Palbociclib and Paclitaxel was detected by distinctive proliferation rates as compared to control conditions. The established ovary cancer-on-a-chip platform enables the study of fundamental aspects of tumor disease and progression. In addition, these co-cultures serve as a platform for understanding tumor-endothelial cell crosstalk and its consequences for tumor aggressiveness. Moreover, these models constitute a suitable platform for drug screenings of anti-cancer and anti-angiogenic compounds, making them a powerful tool for drug selection in personalized medicine applications.

P1795/B33

Quantitation of Interleukin-10 Using a Novel Enzyme-linked Darkening Assay.

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Antibody-based immunoassays are powerful tools used throughout the life sciences including drug discovery, diagnostic tests, and academic research in the STEM fields. This paper introduces the enzyme-linked darkening assay (ELDA), a novel hydrogel particle-based suspension array immunoassay that offers ease of use, adaptability to ELISA antibodies, a large capture area, and does not require expensive instruments for detection. ELDA achieves this by encapsulating the immunoassay complex and the enzymatic product within porous hydrogel beads. The beads are prepared using a proprietary method in which the mesh size and rigidity can be controlled. In an ELDA assay, the capture antibodies are

immobilized in the beads' inner matrix by anchors. As these "capture" beads are incubated with antigen, the antigen molecules bind to the capture antibodies and accumulate within the beads, which are then incubated with AP-conjugated detection antibodies and detection reagent. The reporter enzyme converts the soluble substrate into an insoluble product that is trapped within the matrix of the beads with picoliter volumes, resulting in progressive darkening of the beads based on antigen concentration. Unlike most particle-based immunoassays that measure fluorescence as signal outputs, the optical darkness of ELDA beads is measured as a function of antigen concentration using brightfield microscopy and computer vision detection, and can be imaged using Bioelectronica's proprietary lensless imaging system. The computer vision workflow includes image processing methods to locate each bead and determine the grayscale intensity of the bead centroid region. The rapid imaging of thousands of beads provides grayscale distributions and statistically rigorous confidence intervals. As an initial proof of concept experiment, we demonstrate here that the ELDA assay can detect IL-10, a major inflammation biomarker, using standards prepared at concentrations of 48, 480, and 4800 pg/mL with a total hands on time of <2 hrs. The limits of detection for a variety of biomarkers are still under investigation.

P1796/B34

Improvement of High Throughput Cardiotoxicity Testing System Using Multielectrode Array with Agarose Microchambers.

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Drugs always have side effects, so that toxicity testing is indispensable for drug discovery research. In particular, cardiotoxicity causes arrhythmia, so that the cardiotoxicity testing is the most important item for drug discovery. Since the toxicity testing takes a huge amount of time and cost, a method that further improves cost, ethics, and technical aspects are required. To overcome these problems, a multi-electrode array (MEA) system has been used for cardiotoxicity testing. In the MEA system, cells are cultured on a dish with 64 electrodes, and extracellular potential can be measured for long term without damaging cells. In the conventional cardiotoxicity testing on MEA, only one data was obtained from one dish. Therefore, we aimed to construct a high throughput cardiotoxicity testing system capable of taking 64 pieces of data in one dish by adhering cells only on each electrode. As a method, we used the agarose microchamber (AMC) technology which can control the cell adhesion site. For automated AMC making on each electrode, we investigated the parameters for irradiation of laser (e.g., focal distance, output, and irradiation time). As a result, when the laser focal length was shifted 115 μm in the Z-axis, and the laser output was 4.8 mW on focus, processing by direct laser irradiation to the electrode part as possible. Besides, two-parameter, the laser irradiation time and the processed portion area are related to positive correlation, hence to control the area of the processed portion by controlling the laser irradiation time is possible. Hence, by using this technique, individual cardiomyocyte clusters adhered to only around every 64 electrodes on the MEA chip. The efficiency of cell adhesion was above 95 % (n=4) and the efficiency of cell beating was above 90 % (DIV 3). The extracellular potential on each electrode was simultaneously measured at multiple points. Now we analyze the wave formation of extracellular potential on each electrode and measure the beating rate and field potential duration like QT interval of real heart. In the future, we will validate the efficacy of this high throughput cardiotoxicity testing by applying the several drugs (e.g., E-4031, verapamil, and astemizole).

P1797/B35

Automated Tracking of *S. Pombe* Spindle Elongation Dynamics.

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The mitotic spindle is a microtubule-based machine that pulls the two identical sets of chromosomes to opposite ends of the cell during cell division. Improper spindle mechanics can lead to the chromosomes failing to segregate correctly, which is associated with cancer, miscarriage, and birth defects. The fission yeast Schizosaccharomyces pombe is a popular model organism for studying mitosis due to the simple, stereotyped spindle structure and genetic tractability. S. pombe spindle length is a useful metric for spindle progression, but manually tracking spindle ends in each frame to measure spindle length over time is laborious and can limit experimental efficiency. We have developed an ImageJ plugin that can automatically track S. pombe spindle length over time and replace manual tracking of spindle elongation dynamics. Using an algorithm that detects the principal axis of the spindle and then finds its ends, we reliably track the length and angle of the spindle as the cell divides. The plugin both integrates with existing ImageJ features and exports its data for further analysis outside of ImageJ, and does not require any programming by the user. In the future, we plan to expand the scope to include other applications, such as tracking the poles of mammalian spindles and tracking filament ends for applications such as in vitro motility assays.

P1798/B36

Combining Live Cell Imaging with Cellular Impedance to Monitor Apoptotic Cell Death in Real Time.

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Essential to diverse biological processes such as embryonic development and wound healing, apoptosis (also known as programmed cell death) is an evolutionarily conserved process that enables multicellular organisms to eliminate cells without triggering an inflammatory response. Once triggered by intrinsic or extrinsic stimuli, apoptosis causes a broad array of biochemical and morphological modifications ranging from cytoskeleton degradation and chromatin cleavage to protein cross-linking and phospholipid translocation. The net result of these events is fragmentation of the cell into apoptotic bodies that are rapidly phagocytosed by macrophages. Although apoptosis can be tracked using various biomarker-based assays, these typically require multiple manual handling steps and only yield endpoint measurements. Requiring just a cell seeding step and a drug addition step, we used the Agilent xCELLigence RTCA eSight to continuously monitor drug-mediated apoptosis over the course of multiple days. Providing a direct and objective assessment of cell number, cell size, cell-substrate attachment strength, and cell barrier function, impedance biosensors embedded within the base of eSight microplates are shown to quantitatively track early (cell shrinkage) to late (fragmentation) apoptotic events with high analytical sensitivity. Concurrently, eSight captures live cell images in brightfield and three fluorescence channels (red, green, and blue), providing an orthogonal readout of apoptosis processes such as caspase 3 activation and phosphatidylserine translocation. By combining the strengths of real-time impedance monitoring (simplicity, analytical sensitivity, and objectivity) with that of live cell imaging (specificity of the readout), eSight is shown to increase the information richness of the apoptosis assay without increasing the workload. Importantly, the drug EC50 values determined using these two approaches are nearly identical, suggesting that eSight can simultaneously provide both the primary and secondary (confirmatory) readouts for apoptosis studies.

P1799/B37

Proliferation of Induced Pluripotent Stem Cells Can Be Evaluated by Measuring Exosome Component in Cell Culture Supernatant.

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Quantitative evaluation of cell conditions during cell culture is important for improvement of cell production efficacy. In the field of cell manufacturing, the major cell growth verification method is optical microscope observation so far. An other aproach is metabolites (glucose, lactate and so on) measurement in cell culture supernatant. However, these techniques are not sufficient to grasp cell conditions because microscope images or metabolites do not necessarily accurately reflect cell conditions. In recent years, exosomes, functional vesicles released into blood circulation, have attracted attention as cancer biomarkers. Because exosomes possess wide variety of information of source cells, we expect them as effective index of cell culture conditions. In this study, we demonstrated evaluation of exosomes secreted into cell culture supernatant to verify over time alteration of exosome components (protein expression of CD63 and gene expression of GAPDH) during induced pluripotent stem (iPS) cell proliferation. First, iPS cells (201B7-Ff) were cultured for 7 days and cell culture supernatant was obtained every 24 hours. Cells were harvested and cell number was counted. Then cell culture supernatant was ultracentrifuged, the pellet was observed by transmission electron microscope and particle size distribution was analyzed by resistive pulse sensing. As the results, we found spherical granules and particle size was from 50 nm to 100 nm, the peak of distribution was about 70 nm. Next, we analyzed exosome specific marker expression of CD63 in cell culture supernatant by ELISA. We also quantified GAPDH gene expression ,which is known to be expressed in exosome stably, by quantitative RT-PCR. As the results, expression level of CD63 in cell culture supernatant was increased over time and highly correlated with cell number (R=0.958). Gene expression level of GAPDH was also increased over time and highly correlated with cell number (R=0.955). We showed that iPS cells secrete exosomes into cell culture supernatant by morphological and expression analyses. The expression of CD63, one of exosome markers, and GAPDH expression correlated with cell number, which indicated that these exosome derived signals function as index of cell proliferation. We conclude that exosome can be a novel evaluation index not only for cancer diagnostics, but also for cell conditions during cell culture. This research was partly supported by the Research and Development Grants, Kobe Medical Innovation Cluster.

P1800/B38

Fast and Efficient DNA Transfection in Hard-to-transfect Cells.

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DNA transfection remains challenging for scientists working with primary cells, stem cells and particular cell lines. This is mainly due to the fragility and slow-dividing rates of these cells. Considering the limiting steps in these hard-to-transfect cell types and based on our knowledge and expertise in transfection, we screened a proprietary chemical compounds library. Lead molecules were selected based on their superior transfection efficiencies while maintaining excellent cell viability in different hard to transfect cell types, leading to the development of a novel DNA transfection reagent, jetOPTIMUS®. This reagent

improves cellular uptake and endosomal escape of DNA resulting in higher gene expression in cells. jetOPTIMUS® combines higher transfection efficiency and lower toxicity compared to other commercially available delivery solutions.

P1801/B39

New Strategies for Quality Control in Cell-based Assays.

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New strategies for quality control in cell-based assays Despite the increased focus on sophisticated analysis and advanced cell culture platforms, many of the more traditional assays in 2D format remain central to the research that underpins drug discovery. Conventional techniques used to monitor cell cultures samples are often subjective or destructive, which can lead to erroneous results downstream. Cell counting and confluency analysis are especially important quality controls in cell-based assays, for example to prepare cells for transfections or to prepare samples for downstream experiments. Here we optimize cell culture quality control methods by developing a label-free, trypsinization-free automated process to calculate confluency, cell count, and transfection efficiency directly on the bench. By using cell culture apps, coupled with a cell imager (InCellis, Bertin Technologies, France), we can perform accurate measurements to qualify cell culture sample in order to verify optimal handling, determine cell health and integrity, and collect data. We then validated this method in several experimental contexts from cell proliferation studies, drug assays, cell migration studies, to cell viability assays. We showed that automated calculations of confluency, cell count and transfection efficiency reduces human error and improves overall data integrity.

P1802/B40

Development of a Novel Tunable Synthetic 3D Hydrogel Platform for the Study of Tumor and Stromal Cell Interactions.

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Numerous cancer cell models exist used to investigate disease mechanisms and to screen potential cancer therapeutics. Roughly 90% of promising preclinical drugs fail to result in efficacious human treatments. Traditional two-dimensional (2D) tissue culture models lack realistic complexity, while animal models are expensive, time consuming, and too frequently fail to reflect human tumor biology. Recently, three-dimensional (3D) cell culture models are a new method to generate new drug candidates before moving to expensive and time-consuming animal models. We have developed a biochemically defined hydrogel platform formed by mixing various polymers with chemical crosslinkers with enhanced functionality. The hydrogel system employs one of two types of backbone polymers: a synthetic non-degradable polyvinyl alcohol (PVA) or an enzymatically-degradable dextran. Both polymers are functionalized either with fast or slow thiol-reactive groups. Crosslinkers consist of either PEG non-cell-degradable or a CD cell-degradable crosslinkers containing peptide sequences that create cleavage sites for matrix metalloproteases (MMPs) that allow cell migration. The technology provides mechanical and biochemical cues to investigate both morphological and physiological properties of cells in a 3D environment. The hydrogel allows precise control over hydrogel stiffness, gelation speed, cell migration and allows cell recovery for downstream applications. Here, we demonstrate the utility of this hydrogel platform to grow epithelial, fibroblast and tumor cells in 3D cell cultures with high cell viabilities and functionalities. Furthermore, we have constructed a 3D tumor/stromal cell co-culture

using the hydrogel to model the dynamic tumor cell microenvironment. This technology will allow the creation of more accurate 3D cancer cell models for basic research and drug discovery applications.

P1803/B41

A Novel Method for Total Protein Normalization in Western Blotting That Avoids Invalid Results Obtained When Using Housekeeping Proteins.

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Protein normalization for westerns has relied upon housekeeping proteins which exhibit signal saturation and cellular expression level variations under studied conditions. Consequently, employing housekeeping proteins for normalization of target proteins produces spurious results that lead to erroneous conclusions. A superior method to protein normalization using housekeeping proteins is Total Protein Normalization, a method now recognized as the gold standard for quantitative westerns. Total Protein Normalization requires that proteins on a membrane be stained or labeled uniformly, imaged, and then analyzed for total protein. It is important that such a normalization process not interfere with typical immunodetection methods, fits within existing western workflows, and shows a linear relationship of signal intensity to protein load under all experimental conditions. We have developed a new reagent enabling Total Protein Normalization, and, with this reagent, we demonstrate superior protein normalization capabilities through our analysis of four target proteins in HeLa, MCF-7, Jurkat, and A431 cell lines. Our data illustrate how three housekeeping proteins show signal saturation, yield erroneous normalization data, and display greater than 50% variation. Conversely, signal intensities obtained using our new method show a linear relationship to protein sample load, thereby providing accurate protein normalizations with less than 8% variation. In conclusion, utilization of housekeeping proteins for protein normalization leads to errors in quantifying westerns and subsequently invalid conclusions from experimental studies, while our new Total Protein Normalization method provides an elegant alternative for achieving accurate quantitative westerns.

P1804/B42

Separation of Cells Via Dielectrophoresis on a Novel, Integrated, Benchtop Platform (Cyto R1): Impact of Assay Conditions on Cell Viability.

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Tumors are dynamic environments that contain a variety of resident and recruited cells with changing phenotypes. Their phenotype and interactions are increasingly the focus of research since they determine cancer cell survival and progression. We have developed an integrated benchtop platform (Cyto R1^{TM}) to separate and enrich cells from heterogeneous cell mixtures via contactless dielectrophoresis (DEP) that uses the motion of polarizable particles in a spatially non-uniform electric field to electronically interrogate and discriminate between different cells. Using unique electrophysiological properties of cells rather than protein markers, our goal is to generate enriched viable cell subpopulations for further analyses. Thus, we investigated the viability of various cell lines by exposing the cells to the buffers necessary for the Cyto R1^{TM} platform and electronic stimulation over time and determining cell numbers 24h after plating by MTT. The traditional cDEP buffer reduced cell viability after just 1 hour of exposure by 20-45% for all cell lines, and declined further after 3 hours of exposure (<50%). Cancer cells were more resistant to this treatment than benign epithelial and stromal

cells. We then altered the traditional DEP buffer formulation to balance conductivity, osmolality, and pH to physiologically relevant values (Cyto Buffer A™). Exposing the most sensitive, benign cell line to the Cyto Buffer A™ significantly ($p<0.0001$) increased viability to at least 65% for 3 hours. Using this improved reagent, benign and cancer cell lines were exposed to Cyto Buffer A™ for 2h while undergoing electrical field stimulation on the Cyto R1™ platform. Our results indicate that benign cell lines respond at 628 Vpp whereas cancer cell lines do not respond until 640 Vpp for given frequencies ranging from 200-400kHz indicating separation is feasible on the Cyto R1 ™ platform using the new buffer. Cells exposed to both Cyto Buffer A™ and electrical field stimulations were then resuspended in tissue culture media and plated for growth. After 48 hours, all cells had successfully grown at known doubling rates. Together, our results indicate that the Cyto R1™ system using Cyto Buffer A™ can successfully discriminate between benign and cancer cell lines while sustaining significant cell viability and functionality after 2-3 hours of experimental exposure.

P1805/B43

Functional Recombinant Hydroxysteroid 17-beta Dehydrogenases (HSD17Bs) Produced in HEK293 Cells.

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Hydroxysteroid 17-beta dehydrogenases (HSD17Bs) catalyze oxidation/ reduction of various metabolites generated in sex steroid hormone biogenesis, neurosteroid hormone, retinoid, or cholesterol and fatty acid metabolisms. Studies on genetic variants have indicated HSD17B3, HSD17B4, HSD17B9, HSD17B10 and HSD17B13 as causative factors of human disorders including chronic liver disease. Pathogenic variants of HSD17B and researches on sex-hormone dependent cancers, osteoporosis, and Alzheimer disease suggest that HSD17Bs are potential therapeutic targets. The purpose of the present study was to produce the functionally proven recombinant protein for a better understanding of the biological functions of HSD17Bs and discovering the potential therapeutic drugs of the associated human diseases. HSD17B recombinant proteins expressed in human HEK293 cells were purified using affinity column chromatography. The purity was confirmed by SDS-PAGE and the functional activity was validated by oxidoreductase luminescent assays. This study demonstrated that HSD17Bs produced in HEK293 cells can catalyze oxidoreductive reaction on steroid substrates, which is monitored by concurrent changes in NAD(P)H quantity. In a HSD17B13 kinetics study, it is evidenced that leukotriene B3 is as good as β-estradiol being a HSD17B13 substrate. In addition, the protein sequencing using purified HSD17B13 also addresses the role of the proposed N-terminus signal peptide in its subcellular localization. OriGene Technologies developed over 10,000 full-length human proteins, including HSD17Bs, that are produced in human HEK293 cells, in which the protein structure and post-translational modifications are well preserved. This study suggests that OriGene recombinant HSD17Bs are biologically active and can meet academic and industrial standards in drug discovery.

P1806/B44

Automated Micro-patterning for the Generation of Large Living Cell Arrays on Soft Hydrogels of Adjustable Stiffness.

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The different cellular mechanisms that underlie cell-to-cell and cell-ECM interactions can be studied in *vitro* by controlling cellular microenvironment based on ECM structuration and substrate stiffness. However, current tools for generating cell adhesion microstructures on different biocompatible materials of adjustable stiffness are limited to small surfaces and not really automated. Here, we present an innovative technological tool for the automated generation of large cell arrays applicable to supporting layers of different stiffness. This technological process is based on microcontact printing techniques allowing to automatically produce large arrays (1cm²) of living cells (40 000 cells) immobilized on printed biomolecular micropatterns on microscope glass slides and Polyacylamide (PAA) hydrogels with adjustable mechanical stiffness (Young's modulus from 0.5 to 40 kPa). Different cell lines (PC3, HEK, HeLa) were cultured on this kind of micro-patterned surfaces. The pattern homogeneity and cell spreading inside/outside the defined patterns were statistically analyzed with unprecedented precision thanks to the large population of cells immobilized. Results show excellent conformation of the living cells to the adhesive patterns of different shapes (square, circle and triangle). The square patterns exhibited the highest quality in terms of homogeneity and dimensional control, having a percentage of size variation of only 5.7%. When using these substrates with human cell lines (HeLa), the rate of occupancy of the micropatterns of 20µm size by living cells was found to vary from sample to sample between 38% and 76% with 90% of them accommodating a single cell. This study also highlights the possibility to obtain living cell arrays on PAA films with a bio relevant mechanical stiffness between 0.5 to 40kPa and to conserve the pattern homogeneity on these substrates of choice for the investigation of mechanobiology. This new approach shows for the first time the easy and automated fabrication of large cell arrays of individual living cells on a soft hydrogel material. This innovative bioengineering tool can be applied for the investigation of complex cellular mechanisms at the single cell level but compatible with a large number of observations for statistical consistency.

P1807/B45

Electroporation as a Post-embryonic Delivery Tool for Uncovering Mechanisms of an nelid Regeneration.

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Post-embryonic tools are necessary for testing gene function in juvenile to adult-stage processes such as regeneration yet they are not well-developed for most animal models that regenerate. The development of post-embryonic tools would allow studying regeneration in *i)* organisms when traditional early embryonic manipulation of a gene is lethal due to requirement in embryogenesis, *ii)* in species with long generation times, and/or *iii)* inaccessible embryonic stages (e.g. In animals that reproduce primarily asexually). Electroporation is a widely-used method for delivering large molecules into cells via temporary pores in the cell membrane created by electrical pulses. The technique has already been successfully used as a post-embryonic delivery tool in some regenerative organisms such as the axolotl and zebrafish. This ongoing study aims to develop electroporation as a viable tool for post-embryonic reagent delivery into cells to test gene function during regeneration in annelids; a highly

regenerative group of organisms. To date, we have found that regeneration blastema injection followed by electroporation in *Platynereis dumerilii* (a marine annelid) and *Lumbriculus variegatus* (a freshwater annelid) yielded high uptake of fluorescent-dextran complexes into tissues and individual cells in the blastema, with little to no uptake in injection-only controls. Our future objective is to test delivery of other reagents (ie. morpholinos, siRNAs and CRISPR/Cas9) in these annelid species to provide insight into molecular and cellular mechanisms of regeneration. Funding: Hibbitt Fellowship Startup Funds.

P1808/B46

Novel Basal Cell Culture Media Formulations Enable Long-term Storage at Room Temperature.

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Due to its heavy use in many cell biology laboratories, basal cell culture media is often stocked at high levels in cold storage spaces. Not only do these spaces account for approximately 25% of the energy consumption in typical labs, they are also generally shared across multiple users and can easily become overcrowded. To address this problem, we have developed a set of novel cell culture media that can be stored at room temperature without compromising cell growth, health, or function. An alytical shelf life studies have shown these BenchStable™ media retain critical components during room temperature storage for a period of up to 13 months. To confirm these results aligned with the biology of cell culture, 8 commonly-used cell lines were selected to be maintained over multiple passages in both BenchStable and typical catalog formulations. These studies showed that culture in BenchStable media resulted in populations that exhibited equivalent rates of population doubling and viability relative to culture with conventional media. Because BenchStable media is intended to be stored outside of a refrigerator, it is much more likely to be exposed to light than other formulations. To demonstrate the necessity of additional light-protective packaging, cultures of HEK293 were grown in BenchStable media that had been exposed to common laboratory fluorescent lights for ~21 days. This was compared to age-controlled BenchStable media stored in dark conditions. Cells grown in media that had been exposed to light showed a drastic reduction in growth rate, indicating that breakdown of media components had a negative impact on culture performance. Properly protected from light, room-temperature stored BenchStable media perform equivalently to traditional formulations in all applications tested. Transfection, cryopreservation, differentiation and simple apoptosis assays all show uniform results across both media types. To examine potential differences at the gene expression level, mRNA from MCF7 breast cancer cells grown in traditional and BenchStable media was isolated and converted to cDNA. A customized TaqMan PCR assay focusing on the expression of key signal transduction genes showed that there was no significant up- or down-regulation of genes across more than 15 signal transduction pathways. Taken together, our results show that BenchStable media can be substituted for conventional, refrigerated basal media with very little risk to cell culture performance.

P1809/B47

Monitoring the Interactions and Activation of the Her Family of Receptor Tyrosine Kinases Using Multiplex Duolink and Pan Anti-phosphorylation Antibodies.

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Monitoring the interactions and activation of the HER family of receptor tyrosine kinases using multiplex Duolink and pan anti-phosphorylation antibodies Christopher Melm, Cassandra Herring, Jacqueline Day,

Kelly Keys, Jacob Marler, Tracy Adair-Kirk, and Jeffrey Turner MilliporeSigma, 2909 Laclede Ave., St. Louis, MO 63103 USA the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases consists of four members: epidermal growth factor receptor (EGFR or HER1), HER2, HER3, and HER4. Upon stimulation by different ligands that contain an epidermal growth factor (EGF)-like domain (e.g., EGF or neuregulin), these cell surface receptors can form homodimers or heterodimers, become phosphorylated, and signal through AKT, MAPK, and many other pathways that regulate cell differentiation, migration, proliferation, and apoptosis. Many HER family members are over-expressed in various forms of cancer, making them important drug targets. Here we show that Duolink in *situ* proximity ligation assay (PLA) in combination with high-content screening analysis or flow cytometry can provide a sensitive, quantitative method for screening various inhibitors or biologics. EGF-induced EGFR activation and dimerization with HER2 in SKOV3 human ovarian cancer cells in a multiplex PLA were monitored. PLA allowed precise detection and quantification of this protein-protein interaction, translocation, and protein modifications (e.g., phosphorylation of EGFR and HER2), whereas fluorescent imaging alone could not. We also examined the effects of various EGFR-specific inhibitors on EGFR phosphorylation using a site-specific anti-phosphotyrosine antibody paired with an anti-EGFR antibody. In addition, when site-specific modification antibodies are not available, pan anti-modification antibodies could also be used with target-specific antibodies in a PLA, eliminating the need for directed anti-modification antibodies. Furthermore, we monitored the induced activation and interactions between other HER family members (e.g., HER2-HER2, HER2-HER3, and HER2-HER4 dimerization) in multiplex PLA and determined the effects of therapeutic biological antibodies cetuximab (anti-EGFR) and trastuzumab (anti-HER2) on the HER dimerization pattern. In conclusion, multiplex Duolink PLA allows simultaneous detection of up to four protein events within the same sample and creates an ideal method for screening potential therapeutic agents that affect protein expression, protein-protein interactions, and/or protein modifications.

P1810/B48

Utilizing Fluorescent-based Technology Detecting Protein-protein Interaction (Fluoppi) System to Characterize Targeted Protein Degraders in Cells.

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Inducing the removal of proteins including undruggable targets through targeted protein degradation (TPD) has become a promising approach for novel anti-cancer therapeutics. TPD molecules are bifunctional small molecules that recruit E3 ligases to their target proteins, followed by the initiation of ubiquitination and the degradation of target proteins through the ubiquitin-proteasome pathway. Therefore, TPD molecule-induced intracellular ternary complex, composed of target proteins, E3 ligases, and degraders themselves, is indispensable for the degradation abilities of TPD molecules. However, the intracellular dynamics of ternary complex formation remains unclear. To overcome this problem, we utilized the Fluorescent-based technology detecting Protein-Protein Interaction (Fluoppi) system, which can visualize the protein-protein complex as detectable fluorescent foci in live and fixed cells. Via confocal microscopy or live-cell imaging, we here demonstrate that bromodomain and extra-terminal (BET) protein degraders induced specific foci in dose- and time-dependent manners in cells coexpressing Fluoppi-tagged BRD4 and E3 ligase, but their moieties did not. Intriguingly, these specific foci were seen in the region of the cell nucleus where BRD4 was localized, suggesting that the Fluoppi system can be used to clarify where the TPD molecules-induced ternary complex occurs. We further demonstrate that the kinetics of the formation of foci induced by BET protein degraders correlated with that of the levels

of target protein degradation. Taking these findings together, we propose that the Fluoppi system could be a useful research tool for validating TPD molecules by visualizing the spatiotemporal formation of the ternary complex in cells.

P1811/B49

Active Patch for Transdermal Drug Delivery and Beauty Treatment Using Photoreactionbased Microcurrent.

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This presentation reports a photoreactive micro-current generation patch that can be used for a beauty treatment system and transdermal drug delivery (TDD), and more specifically to an element including a drug composition and the application of the element to therapeutic and beauty treatment applications. A normal transdermal electrical potential is present in the skin of a human body. The fact that current is important to the maintenance of the healthy skin can be seen from the successful results of electrotherapy in the treatment of the damaged skins. Electrophoretic treatment designed to apply a low-intensity direct current (DC) flow to ulcer sites reduced the pain and discomfort of ulcers and improved cure rate. A DC electric field is effective in the processes of ligament fibroblast migration and wound healing, and cutaneous lesions are stimulated by external current in the treatment thereof. Accordingly, the maintenance of current in the skin is important for the sustained maintenance of the intact skin. Micro-current patches for transdermal drug delivery and beauty treatment, which are each characterized by a considerably thin power supply and electrodes printed or stacked on a flexible plastic substrate have emerged in recent years, and have been used for active beauty treatment or transdermal drug delivery. This presentation explains the active patch that generates micro-current through the photoreaction of the zinc oxide part obtained by combining zinc oxide and cellulose as long as light is radiated and allows the micro-current to continuously flow to the skin through the drug carrier, thereby achieving transdermal drug delivery and skin care effects. This patch is flexible, self-powered and convenient to attach on skin. The concept and test results of the patch will be explained.

P1812/B50

Dual Antigen Capture and Detect. Evix Tools for Sorting and Phenotyping Ev Populations.

M. Wolf, J. Orf, P. Everson; InCepix, St. Paul, MN.

Determining the physiological role of extra-cellular vesicles (EVs) requires identification and characterization of proteins associated with each type of vesicle. Using EVix tools to capture antigen specific EVs (Capture) followed by detecting these structures in a subsequent antigen specific assay (Detect) we report on the variety of specific EVs isolated from human urine. EVix tools allow for simple and sensitive immune-phenotyping of human EV populations and reveal unique surface antigen population profiles. Normal human urine EVs present CD10 and other cell surface enzymes and specific markers (CD13, CD24, CD 133, CD147 and CD26) in unique combinations. EVix tools reveal unique distribution patterns of human EV specific antigens when samples are characterized on size exclusion chromatography columns Capture with cholera B toxin generates unique detection antigen profiles. Examination of specifically captured EVs demonstrates the presence of unique populations of EVs within the sample. We show that EpCAM (CD326) is present on normal urine EVs and EVs containing CD326 can be specifically captured and characterized for the presence of other antigens. We show that capture of specific antigen is critical in uncovering certain rare or less prevalent subpopulations within the overall

EV population. Capture strategies relying on tetraspan specific antigens (CD9, CD63, CD81) were not successful in revealing EpCAM (CD326) positive EVs. This is most likely because tetraspan positive but EpCAM negative EVs obscure the low level signal from the less prevalent EpCAM positive EVs. Our data also suggests that EpCAM (CD326) is present as a single or very low copy number on the EVs captured, unlike Neprilysin (CD10) and other surface markers.

Actin and Actin-Associated Proteins 3

P1813/B52

Rapid Production of Acetylated, Methylated, and Arginylated Actin Isoforms in *Pichia Pastoris*.

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Actins are major eukaryotic cytoskeletal proteins, which perform many important cell functions, including cell division, cell polarity, wound healing, and muscle contraction. Despite obvious drawbacks, muscle actin, which is easily purified, is used extensively presently for biochemical studies of actin cytoskeleton from other organisms / cell types. Previously, we reported a rapid and cost-effective method to purify heterologous actins expressed in the yeast *Pichia pastoris*. Actin was expressed as a fusion with the actin-binding protein thymosin β4 and purified using an affinity tag introduced in the fusion. Following cleavage of thymosin β4 and the affinity tag, highly purified functional full-length actin is liberated. We purify actins from *S. cerevisiae*, *S. pombe*, and the β- and γ- isoforms of human actin. Recent work has highlighted the importance of post-translational modifications in the regulation of actin function. Three modifications have been characterized in detail. 1. N-terminal acetylation 2. N-terminal arginylation and 3. His73 methylation. Recently, we have further developed methods to express in Pichia and allowing us to purify a single actin in the apo-form and bearing five different post-translationally modified forms. To achieve this, we have created Pichia strains expressing human NAA80 acetyl transferase and / or SETD3 methyl transferase. We demonstrate the utility of this synthetic system in the expression of human non-muscle and Arabidopsis actin. Finally, we demonstrate the utility of our method in the purification of actin bearing point mutations, including those that cause human disease. The methods should greatly facilitate biochemical and cell biological studies of the actin cytoskeleton and the effects of post-translational modifications therein.

P1814/B53

Quantification of Intracellular N-terminal Beta Actin Arginylation.

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Actin is a ubiquitous, essential, and highly abundant protein in all eukaryotic cells that underlies muscle contraction, as well as cell adhesion, migration, and leading edge dynamics. The two non-muscle actins, beta and gamma, are ubiquitously present in every cell type and are nearly identical to each other at the amino acid level, but play distinct intracellular roles. The mechanisms regulating this distinction have been the focus of recent interest in the field. Work from our lab has previously shown that beta, but not gamma, actin undergoes N-terminal arginylation on Asp3. While functional evidence suggest that this arginylation may be important to actin's function, progress in these studies so far has been hindered by difficulties in arginylated actin detection, precluding estimations of the abundance of arginylated actin in cells, and its occurrence in different tissues and cell types. The

present study represents the first antibody-based quantification of the percentage of arginylated actin in migratory non-muscle cells under different physiological conditions, as well as in different cells and tissues. We find that while the steady-state level of arginylated actin is relatively low, it is consistently present in vivo, and is somewhat more prominent in migratory cells. Inhibition of N-terminal actin acetylation dramatically increases the intracellular actin arginylation level, suggesting that these two modifications may directly compete in vivo. These findings constitute an essential step in our understanding of actin regulation by arginylation, and in uncovering the dynamic interplay of actin's N-terminal modifications in vivo.

P1815/B54

Essential Roles of Beta-Actin Protein in Maintaining Primary Cillia.

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Lack of cytoplasmic beta-actin gene (Actb) leads to early embryonic lethality in mice, however targeted editing of Actb coding sequence by introducing five point mutations to encode cytoplasmic gamma actin protein does not affect mouse viability. Mice with beta to gamma actin replacement (Actbc-g mice) develop normally and show no detectable phenotypes at young age, despite complete lack of beta actin protein. Here we investigated the effect of the replacement of beta actin with gamma cytoplasmic actin in the retina --- the tissue where these two cytoplasmic actin isoforms predominate. Actbc-g mice developed progressive loss of vision and disorganization of photoreceptor outer segments, a phenotype reminiscent of retinitis pigmentosa, which arises in a variety of ciliopathies. Our data strongly suggest that beta-actin protein is essential for maintaining photoreceptor cilia and normal vision.

P1816/B55

Novel, Rapidly Evolving Actin-Related Proteins Have Acquired Specialized Functions at Critical Actin Structures in the Male Germline.

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Cytoskeletal proteins perform many fundamental biological processes in all eukaryotes, and given their essentially, they are conventionally thought to be highly conserved among most phyla. Actin-related proteins (Arps) are among the cytoskeletal proteins that originated more than a billion years ago in eukaryotes and are thought to be well conserved. However, some Arps in both mammals and flies display evidence of genetic innovation via rapid expansion of gene families and accelerated amino-acid substitutions (positive selection). Rapid genetic innovation is often indicative of an adaptive advantage for sequence diversification. While the canonical Arps are ubiquitously expressed in all tissues, divergent Arps in mammals and *Drosophila* are surprisingly expressed exclusively in the male germline, suggesting they have evolved specialized roles in male reproduction. Given the paucity of information regarding the function of gametic Arps in any species, we sought to understand the role of *D. melanogaster* Arp53D in the testis. We find that Arp53D, which is most closely related to cytoplasmic actin, localizes to two critical germline-specific cytoskeletal structures. First, during male meiosis, Arp53D localizes to the fusome, an actin-enriched membranous organelle that connects all germ cells in a cyst. Second, following spermatid elongation, Arp53D colocalizes with the molecular motor myosin VI at the leading edge of actin cones, unique structures that separate syncytial spermatids into individual cells and push excess cytoplasm to the end of the sperm flagellar tail. We also find that an unusual N-terminal tail, but

not its canonical actin fold domain, is required for Arp53D's novel localization. Furthermore, loss of Arp53D results in reduced organismal fitness, most likely as a result of male subfertility. Taking a broader phylogenomic approach to identify additional cases of genetic innovation among Arps, we discovered a 15-million-year-old expansion of functional Arp paralogs in one clade of *Drosophila.* Intriguingly, similar to Arp53D, the clade-specific Arps are also expressed exclusively in the testis and localize to unique germline cytoskeletal actin structures, including actin cones. Our findings reveal an unexpected, recurrent specialization of divergent Arps for roles at male germline actin structures.

P1817/B56

Tissue Specific Pleiotropy of β- and γ-cytoplasmic Actin Depends Differentially on Nucleotide Or Amino Acid Sequence.

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Many studies over the years have revealed conflicting data regarding the tissue specific roles of highly similar β- and γ- cytoplasmic actins, showing unique and redundant functions which appear to rely on either the nucleotide sequence, amino acid sequence, or both. The two proteins differ by only 4 biochemically similar amino acids but show more variability in the nucleotide sequences. At the organismal level, the β-actin nucleotide sequence, but not protein, is essential for viability. Mice expressing the γ-actin protein sequence from the β-actin locus are almost entirely phenotypically normal, in contrast to β-actin knock-out (KO) mice that are early embryonic lethal. In tissue specific conditional KO models, the two cytoplasmic actins have similar functions. In the stereocilia of the inner ear, β- and γ-actin are redundant in development but have unique functions in stereocilia maintenance with KO causing unique types of progressive hearing loss. Muscle specific KO of either isoform causes a mild, but progressive myopathy. A primary mouse embryonic fibroblast (MEF) model shows that β-actin KO causes loss of proliferation and severely impaired motility while γ-actin KO MEFs present with a smaller, but significant decrease in proliferation and normal motility. Here, we attempted to understand why the primary MEFs were so severely affected by β-actin KO. Using qRT-PCR we assayed for expression of truncated *Actb* or *Actg1* transcripts, deleted for exons 2 and 3 but encoding exons 4-6, to mirror the mouse KO models. β-actin expression was 86 fold higher than γ-actin in control cells, and increased to 314 fold for truncated β-actin transcript in the KO cells. Because the β-actin KO cells have such severe phenotypes and γ-actin KOs do not, the greatly increased expression of the truncated β-actin transcript may be toxic. Our analysis also revealed a significant, 3.5 fold increase in expression of the truncated transcript from the β-actin locus in primary MEFs and an insignificant increase in immortalized MEFs. These results are of interest because only the primary β-actin KO MEFs have failed proliferation and impaired motility, further suggesting that overexpression of the truncated transcript is toxic. We conducted the same analysis on γ-actin KO MEFs and β- and γ-actin KO muscle, but measured a slight, but non-significant increase in expression of the truncated transcripts. Further evaluation of the impact of overexpression of exogenous β-actin transcript deleted for exons 2-3 will help uncover the cause of the variable mechanisms of pathogenesis resulting from dysregulated cytoplasmic actin expression.

P1818/B57

The Role of Clp36 in Pancreatic Cancer Cells during Migration and Cell Shape Change.

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Cancer is a disease characterized by uncontrolled cell proliferation, migration, invasion, and morphology. Actin and alpha-actinins (ACTNs) are key players that underpin these cellular processes. Due to the overexpression of ACTN4 and its adaptor protein CLP36 in other cancers, we hypothesized that these proteins might contribute to migration, cell morphology, and mechanoresponsiveness of pancreatic cancer cells. We assessed CLP36 expression and found that CLP36 is highly expressed in stage IV ascites-metastasis-derived AsPC-1 cells, but less so in immortalized human pancreatic ductal epithelial HPDE, stage II pancreatic adenocarcinoma-derived Panc10.05, colorectal carcinoma-derived HCT116, and HeLa cells. We then knocked down CLP36 using shRNA in AsPC-1 cells and used scratch wound assays to test whether CLP36 is necessary for cell migration. In two knockdown cell-lines, removal of CLP36 resulted into significantly decreased cell motility. Immunofluorescence was then performed on AsPC-1 cells, and we found that CLP36 and actin filaments co-localized at the cell cortex. In contrast, in HPDE cells, CLP36 was distributed perinuclearly, while actin remained cortical, while in Panc10.05 cells, CLP36 was largely cytoplasmic. We are now exploring how CLP36 contributes to alpha-actinin-4's role in cell mechanics and mechanoresponsiveness. Overall, our results already suggest CLP36 is an important component of pancreatic cancer cell migration and may contribute to cancer progression.

P1819/B58

Tropomyosin Isoforms Expression in Healthy and Injured Kidney Podocytes.

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The podocytopathies are a group of glomerular diseases that affect the kidney's ability to filter the blood, and often lead to kidney failure. To help establish and maintain the filtration barrier, healthy podocytes cover the glomerular capillaries with thousands of extensions called foot processes that interdigitate with one another and are joined by a special cell-cell junction, the slit diaphragm. Podocytes maintain their elaborate cell shape by tightly regulating their actin cytoskeleton, which is linked to the slit diaphragms. Interestingly, podocytes respond to insults in a stereotypical fashion by undergoing foot process effacement (FPE), a dramatic shift in podocyte morphology and disappearance of the intricate foot processes. Tropomyosins (Tpms) are coiled-coil dimers that form co-polymers along actin filaments and change the filaments' biophysical properties. Over 40 different Tpm isoforms have been identified as the gene products of 4 *Tpm* genes: *Tpm1*, *2*, *3* and *4*. Various Tpm isoforms target to different locations inside cells and change the type of actin cables assembled in those locations. We hypothesize that podocyte shape is controlled by a specific set of Tpm isoforms that regulate actin cytoskeletal dynamics. Changing the tropomyosin isoforms after injury might be linked to changing podocyte shape and the FPE phenomenon. To test that, we used RT-PCR and immunoblotting and identified various Tpm isoforms as enriched in podocyte cell lines and in isolated healthy glomeruli. Using different mouse models for podocyte injury (i.e., *Cd2ap* KO, *Lamb2* KO, *Col4a3* KO and Adriamycin-nephropathy (AdrN)), we identified a change in Tpm isoforms in the glomeruli isolated from these mice. To reveal the entire array of Tpm isoforms in podocytes, we utilized RNAseq using isolated glomeruli to obtain both short reads as well as long-reads using an Illumina and PacBio instruments, respectively. RNAseq results from WT glomeruli show a different pattern of Tpm expression than the counterparts in injured glomeruli, with the most significant changes occurring in Tpm 1.7 and Tpm 3.4.

We next isolated RNA from WT and AdrN glomeruli taken from podocyte-specific translating-ribosome-affinity-purification ("TRAP") mice. By pulling down GFP-tagged ribosomes and purifying the attached mRNA for RNAseq, we were able to purify podocyte mRNA away from that in other glomerular cells types (mesangial and endothelial cells). Comparing the isolated RNA from WT-TRAP and TRAP mice subjected to AdrN, we are able to identify the podocyte-specific Tpm isoforms that are associated with injury. Collectively, this study suggest roles for tropomyosin isoform changes in regulating podocyte shape in health and injury conditions.

P1820/B59

Tropomyosin and Myosin 18a Play Important Roles in the Organization, Dynamics and Function of the Actomyosin Arcs That Form at the T Cell Immunological Synapse.

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T cells are a critical arm of the adaptive immune system because they kill virally-infected or transformed cells and facilitate the function of other immune cells. T cell dysfunction can lead to an array of severe pathologies including susceptibility to infection and autoimmunity. T cell activation requires recognition by the T cell's unique T cell receptor (TCR) of specific peptide antigen bound to major histocompatibility complex (MHC) on the surface of an antigen-presenting cell (APC). This recognition can lead to long-term stable engagement with the APC and the formation of a highly-organized structure at the T cell: APC interface termed the immunological synapse (IS). The IS itself is a multidomain structure divided into distal, peripheral, and central supramolecular activation complexes (dSMAC, pSMAC, cSMAC). TCR microclusters contact antigen-bearing MHC at the periphery of the IS and are then transported across the dSMAC and pSMAC to the cSMAC. This centripetal movement of microclusters is driven by the retrograde flow of an Arp2/3-generated, branched actin network in the dSMAC and the contraction of formin-generated, myosin 2-rich, concentric actin arcs in the pSMAC. Perturbation of either of these actin structures dampens TCR signaling and impairs T cell activation. The goal of this study is to characterize the roles of tropomyosin and myosin 18A in the organization, dynamics and function of the pSMAC actomyosin arcs. Tropomyosins are actin-binding proteins that form head-to-tail polymers along the actin filament. Several tropomyosin isoforms have been shown to associate preferentially with linear, formin-generated filaments, where they promote the recruitment and activation of myosin 2 and thwart cofilin-mediated filament disassembly. In preliminary experiments, we find that the low-molecular weight tropomyosin isoforms 3.1 and 4.2 associate with the pSMAC arcs, and that the tropomyosin inhibitor TR100 disrupts their organization. Myosin 18A is a myosin 2-like protein that lacks motor activity and contains unique N- and C-terminal extensions harboring both recognizable and uncharacterized protein: protein interaction domains. Importantly, myosin 18A co-assembles with myosin 2 to make mixed filaments, suggesting that myosin 18A serves to recruit proteins to these mixed filaments or attach them to cellular structures. Preliminary experiments show that the myosin 18A isoform myosin 18Aβ is highly expressed in T cells and that it co-assembles with myosin 2 in the pSMAC arcs. Moreover, knockdown or knockout of myosin 18A alters arc organization and attenuates proximal signaling. Current efforts are directed at further clarifying the roles played by tropomyosin and myosin 18Aβ in arc organization and function, as well as in T cell effector functions.

P1821/B60

Expression and Characterization of Non-muscle Tropomyosin Isoforms.

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Tropomyosin is a coiled-coil dimer that polymerizes along the length of actin filaments and regulates the binding of other proteins to actin. In muscles, tropomyosin regulates myosin binding and contraction, while in non-muscle cells tropomyosin plays roles in diverse functions such as cell motility, adhesion, cytokinesis, and neuronal development. While there is high sequence similarity among these proteins, in cells they do not compensate for each other upon knockout or overexpression. A major gap in knowledge remains, which is the mechanism that allows specific sets of tropomyosin isoforms to perform specific roles in cells. Previous work in the field has attempted to characterize these isoforms biochemically, but has been inconclusive because of major difficulties obtaining physiologically relevant protein for in *vitro* study. In this work, we have developed a new mammalian-cell expression system that allows specific tropomyosin isoforms to be purified with native post-translational modification and binding partners. We report that all N-termini are acetylated in cells, and that the major isoforms of non-muscle tropomyosin exist in cells as heterodimers. We provide evidence that these heterodimer pairs of tropomyosin exhibit different actin binding affinity and capping activity across isoforms.

P1822/B61

The Actin Crosslinking Protein Filamin, Promotes Cortical Stiffness to Mediate Leader Bleb Based Migration of Melanoma A375 Cells Under Non-adhesive Confinement.

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Within the confines of tissues, cancer cells can use blebs to migrate throughout the interstitium. In confined 3D microenvironments mimicking the tissue microenvironment, cancer cells exhibit plasticity of migration modes depending on the degree of confinement, level of contractility and the availability of ligand adhesion. Under high confinement and contractility and low adhesion, intracellular pressure drives the formation of a large bleb that mediates rapid persistent migration in a process termed ‘leader bleb-based migration’ (LBBM). LBBM is characterized by an unusual, highly polarized cell morphology with a long (~20 um) sausage-shaped bleb that points in the direction of movement, separated from a smaller (~10-15 um) spherical cell body by a contractile neck. LBBM is driven by actomyosin assembly and retrograde flow along the bleb coupled to non-specific friction with the microenvironment. However, neither the fundamental organization of organelles nor the role of actin regulatory proteins mediating this unusual mode of motility is known. We performed a survey of the localization of organelle markers and actin-associated proteins including nucleators, bundlers and crosslinkers in metastatic human A375 melanoma cells undergoing LBBM in non-adhesive confinement. We expressed fluorescent fusion proteins in cells confined to a 3um space under polydimethylsiloxane (PDMS) imaged by time-lapse spinning-disk confocal microscopy to analyze the spatial distribution within the cell body and leader bleb, as well as the frequency of morphology switch and cell motility parameters. Our results indicate that most cytoskeleton and membranous organelles examined are localized in the cell body; however, microtubules and intermediate filaments as well as the Golgi and ER also extend into the leader bleb, peroxisome markers localize near the neck at the base of the bleb, and the nucleus and centrosome translocate between body and bleb. We found that actin nucleators Arp2/3 and mDia2 localizes towards the leader bleb tip, while a/β-spectrin are confined to the cell body and fascin and

filamin are found exclusively in the bleb. An alysis of morphology and motility parameters in cells overexpressing actin regulatory fusion proteins revealed that overexpression of filamin increased bleb size and LBBM speed, while siRNA knockdown of filamin decreased LBBM directionality. AFM analysis showed that filamin overexpression promotes cortical tension. Our study provides the first description of the cellular "anatomy" during LBBM and suggests an important role of actin crosslinkers in regulating leader bleb formation, speed and directionality.

P1823/B62

Cell Protrusion Across Matrix Gaps Requires T-Plastin Actin Bundling Activity.

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To migrate in *vivo*, cells move across diverse assemblies of extracellular matrix (ECM) that can be separated by micron-scale gaps. For membranes to protrude and reattach across a gap, actin filaments, which are relatively weak as single filaments, must polymerize outward from adhesion sites to push membranes towards distant sites of new adhesion. Here, using micropatterned ECMs, we identified T-Plastin, one of the most ancient actin bundling proteins, as an actin stabilizer that promotes membrane protrusions and enables bridging of ECM gaps. We found that T-Plastin is specifically enriched in active protrusions where F-actin is devoid of non-muscle myosin II activity. Additionally, loss of T-Plastin caused defects in lamellipodial architecture without perturbing actin treadmilling rates. Together, our study uncovers critical structural roles of the actin bundler T-Plastin to promote protrusions and migration when adhesion is weak or spatially-gapped and under conditions of high membrane tension.

P1824/B63

Osteoporosis Mutations Lead to Impaired Calcium Regulation of Actin Bundling by Plastin-3.

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Mutations in actin-bundling protein plastin 3 (PLS3) emerged as a cause of osteoporosis, but the underlying mechanisms are unknown. Of the five tested in this study osteoporosis PLS3 mutations not associated with gene deletions/truncations, one distorts an actin-binding loop and abolishes F-actin bundling as revealed by cryo-EM and protein interaction assays. The remaining four mutants retained the bundling ability but showed either decreased or increased sensitivity to Ca^{2+} in reconstituted in vitro assays. Not being located in the regulatory calcium-binding domain, the loops harboring the mutations are protected by the regulatory domain, revealing for the first time its position at the interface of the actin-binding domains and suggesting the mechanism of plastin activity inhibition. In cells, wild-type PLS3 was distributed between lamellipodia and focal adhesions. In contrast, the Ca^{2+}-hypersensitive PLS3 mutants were restricted to lamellipodia, albeit their localization at focal adhesions could be partially rescued upon chelation of extracellular Ca^{2+} by EGTA. Accordingly, the Ca^{2+}-hyposensitive mutants were not found at the leading edge but localized exclusively at focal adhesions/stress fibers, which displayed reinforced morphology. These findings suggest that a mutational disruption of actin bundling or the Ca^{2+}-controlled PLS3's cycling between adhesion complexes and the leading edge leads to severe osteoporosis similar to that caused by the PLS3 deletions. To our knowledge, this study is the first direct evidence that the Ca^{2+}-dependent regulation of plastins is essential for their physiological functions.

P1825/B64

Plastin Promotes Fast Actin Filament Bundling Along with Formin-mediated Polymerization to Generate Rapid Filament Alignment during Contractile Ring Assembly.

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Cytokinesis is the process of physically dividing one cell into two, by means of a contractile ring made up of filamentous actin and non-muscle myosin. A key step in the assembly of the contractile ring is the rapid alignment of actin filaments into an organized array at the equator of the dividing cell. One proposed mechanism for local reorientation of actin filaments is compressive flow in the dividing cell, but whether this flow is sufficient to rapidly and robustly align filaments while the filaments are undergoing constant turnover is unclear. To evaluate what is required for timely assembly of the contractile ring, we used single molecule imaging and particle tracking analysis to measure local filament turnover and cortex contraction rates during the first cell division in the *C. Elegans* embryo. With these measurements and simple mathematical models, we found that compressive flow alone cannot account for the amount of filament alignment we observe during assembly of the contractile ring. Expanding on this model, we found that anisotropic assembly of actin filaments, along with slow realignment of filaments via myosin motor activity, could account for the rapid emergence and maintenance of a circumferentially aligned filament array. To evaluate if this mechanism of alignment operates in *C. elegans*, we imaged embryos expressing CYK-1/formin::GFP and LifeAct::mCherry and found that filament elongation is highly anisotropic at the equator of the cell, but not at the poles. Furthermore, a significant portion of CYK-1-polymerized filaments entering the equatorial region change direction to align with the arrayed filaments in the contractile ring. These results are consistent with templated assembly of actin filaments at the equator. We identified the *C. Elegans* homolog of the actin binding protein plastin (PLST-1) as a likely candidate to promote rapid bundling and alignment of fast-growing CYK-1 filaments in the embryo. Consistent with a role in assembly of the contractile ring, loss of PLST-1 results in slowed or abortive cytokinesis. Using multi-color TIRF microscopy to visualize actin polymerization by fluorescently labeled CYK-1 and crosslinking by fluorescently labeled PLST-1, we found that PLST-1 can rapidly bundle and cause realignment of CYK-1/formin polymerized actin filaments in-line with pre-existing actin filaments, consistent with our findings in *vivo*. Our results demonstrate a role for an actin bundling protein in promoting the templated elongation and alignment of actin filaments in the contractile ring and provide new insights into how the ring is rapidly and robustly assembled during cell division in animal cells.

P1826/B65

The Mechanism of Actin Remodeling by the BAR Domain of ArfGAP ASAP1.

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The actin cytoskeleton plays an important role in cellular processes. Normal processes, such as differentiation, use the actin cytoskeleton as a signaling and transport platform, while aberrant

processes, such as cancer, repurpose actin for invasion. Actin remodeling involves assembly of globular actin into actin filaments and further assembly of filaments into bundles, which form structures such as stress fibers, lamellipodia and filopodia. While actin remodeling activity of actinin and fascin protein families and their importance in higher order actin assemblies are well established, novel families of actin regulators have emerged. Recently, it was shown that a subset of proteins, most extensively studied for inducing or sensing membrane curvature, the BAR domain proteins, can facilitate formation of higher order actin structures. However, not all BAR domain proteins can remodel actin and their exact mechanism in the regulation of the actin cytoskeleton remains unclear. ASAP1 and ACAP1 are BAR-domain containing ArfGAPs of the human ArfGAP family. ASAP1 is an Arf1/5-specific ArfGAP that regulates migration and focal adhesion dynamics. Its effects on actin and focal adhesions are well documented but can only be partially assigned to its role as the regulator of Arf signaling. ACAP1 is an Arf6-specific ArfGAP that regulates integrin recycling and membrane deformation. Here we show that the BAR domain of ASAP1, but not ACAP1, organizes F-actin into bundles to regulate stress fiber maintenance. Overexpression of the BARPH region of ASAP1 led to increase in actin content and appearance of actin spikes at cell edges across three different cell types, indicating that the BAR domain remodels actin. In vitro cosedimentation- and fluorescence-based binding assays showed that the BARPH of ASAP1 and not ACAP1 binds and bundles actin filaments. Further dissection of the mechanism of actin bundling via structural comparison, followed by site-directed mutagenesis of basic clusters, localizes the actin binding site to the 1st and 2nd helix of the BAR domain of ASAP1. Together these data provide evidence for multimodal mechanism of actin remodeling by ASAP1 and highlights selectivity of BAR domains in actin binding.

P1827/B66

ASAP1 Directly Binds to Actin Filaments Via Its N-BAR Domain.

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The Arf GTPase-activating protein ASAP1 localizes to and regulates several actin-based mechanosensitive structures such as focal adhesions, invadopodia, and podosomes. The dynamics and organization of actin filaments are crucial for the assembly, maintenance, and turnover of these structures and the related cellular functions including cell adhesion, migration and invasion. The Arf GAP activity of ASAP1 accounts for some but not all cellular functions of ASAP1. In this study we find that the amino-terminal N-BAR domain of ASAP1 directly binds to F-actin and cross-links actin filaments into thick bundles. The actin bundles induced by the BAR-PH tandem of ASAP1 are predominantly unipolar. Further, the BAR-PH tandem reduces depolymerization of the actin bundles and the spontaneous polymerization of G-actin, indicating a role of ASAP1 in stabilizing F-actin bundles. We also find that overexpression of the BAR-PH tandem of ASAP1 in fibroblasts induces formation of cellular projections more effectively than full length ASAP1 whereas ASAP1 lacking the BAR-PH domain fails to induce these structures. Together our data support a model in which ASAP1 regulates the dynamics and higher order structures of actin cytoskeleton, at least in part, by direct binding to F-actin and the effect on actin dynamics is controlled by domains outside of the N-BAR domain. To our knowledge, this is the first report of an Arf GTPase-activating protein that directly interacts with F-actin.

P1828/B67

Role of the Size of Actin Crosslinkers in Actomyosin Cortex Tension Regulation.

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Controlled cell shape changes are key during fundamental processes such as division, migration, and epithelial morphogenesis. In animal cells, many shape changes are driven by the actin cortex, a thin, highly crosslinked actomyosin network that acts as a scaffold for the plasma membrane. The actin cortex is under contractile tension and tension gradients lead to local contractions that result in cell shape changes. It is thus crucial to understand how cortical tension is regulated. Cortex tension is primarily generated by myosin motors pulling on actin filaments. To date, most studies of cortical tension regulation have focused on the role of myosin activity. However, recent studies have highlighted that the organization of the actin network itself is central to cortical tension control. In particular, several studies indicate that changing the levels of various actin crosslinkers, proteins that can bind two actin filaments to each other, can change cortical tension. There are over 20 different actin crosslinkers in the cortex. Interestingly, these crosslinkers greatly vary in size, ranging from ~5 to >100 nm. This project aims to investigate the role of the size of the actin crosslinkers in actomyosin cortex mechanics. To identify the crosslinkers most important in cortex tension regulation we optimised a protocol to isolate cortex-enriched blebs. This allowed us to isolate the actin cortex from cells in different phases of the cell cycle, where cells display different levels of cortical tension. Mass spectrometry of these cortical fractions allowed us to select a subset of potential key crosslinkers involved in cortical tension regulation in an unbiased manner. We then investigated the localisation within the cortex of these different crosslinkers. The actin cortex is only ~200 nm thick in mitotic cells. Thus, super-resolution microscopy is required to investigate the relationship between the size and localization of the actin crosslinkers within the cortex. We use dual colour dSTORM for multi-colour super-resolution visualization of actin cortex and actin binding proteins. Furthermore, to investigate the role of size independently of other features, we developed artificial crosslinkers. The artificial crosslinkers only vary in size while maintaining other structural properties the same. Together, this study will unveil to what extent size affects crosslinker localisation within the cortex, allowing us to explore how cortex structural arrangement affects cortical tension.

P1829/B68

Traction Force Microscopy Reveals Unique Stress Profiles for Cells in the Absence of Arp2/3 and Fascin.

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The cytoskeleton and its associated proteins are responsible for force generation and a number of crucial cellular functions including homeostasis, development, and motility. In particular, the cytoskeletal players Arp2/3 and Fascin, have been demonstrated to be crucial for sensing gradients of fibronectin, also known as haptotaxis migration. In order to better understand the role these proteins play in cellular motility and force generation, we require a method of probing cell behavior in the absence of these proteins. Using a system of PAA (Poly-Acrylamide) soft substrate gels embedded with

fluorescent reporter beads localized to the surface, we are able to observe cells exerting stresses and collect traction force data inferred from the displacement of beads. We have used this technique to measure the traction forces and total strain energy exerted by a mouse embryonic fibroblast cell line. We have optimized a MATLAB code to account for increased throughput and data processing by automatic calculations of cellular area and data statistics following traction field generation. Using these methods, we observed that cells plated on PAA substrates of differing stiffness exert traction forces that display a characteristic trend; while bead displacements decrease as stiffness of the gel increases, the average traction force of the cell increases. Furthermore, we have compared the traction forces exerted by Arp2/3 null and Fascin depleted cells to that of wild type fibroblasts. ARP2/3 null cells display a decreased average traction force while maintaining a strain energy density not significantly different to wild type cells. Fascin null cells display a similar trend. Additionally, the traction force profile for the depleted cells show remarkable differences from their wild type counterparts. These characteristic traction force maps were correlated to the underlying mechanisms of cell motility using a GFP Paxilin cell line. An examination of the traction forces resulting from a change in these key cytoskeletal proteins may provide a better understanding of how these molecular players contribute to directed migration.

P1830/B69

Differential Contribution of Actin Filament Bundling Proteins to Filopodia Formation and Maintenance.

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Filopodia are dynamic protrusions extending from the cell periphery acting as sensory organelles in migration, adhesion, neuronal guidance and wound healing. They are linked to pathological conditions, such as cancer and viral infections. Filopodia consist of actin filaments spatially organized into bundles by actin bundling proteins, various combinations of which are expressed in different cell types. The reasons for the presence of multiple filament bundling proteins in filopodia and of varying combinations of them in different cell types and physiological contexts, however, are unknown. We therefore decided to define their relative contributions to filopodia formation in a systematic fashion. We employed CRISPR/Cas9-mediated gene disruption in B16-F1 mouse melanoma cells to eliminate expression of Fascin, T-Plastin or Daam1 proteins, all previously reported to participate in actin filament bundling in this cell type. We quantified density, length as well as morphological features such as straightness of filopodia and microspikes (actin bundles sharing filopodia-like characteristics, which are not protruding beyond the cell periphery). (Cryo)-electron tomography was employed to examine the ultrastructure of actin filament arrays within filopodia and microspikes. Our results indicate that no single actin filament bundling protein is essential for filopodial initiation. Whereas removal of Daam1 did not lead to major defects in filopodia numbers or morphology, depletion of T-plastin modestly reduced the number of longer filopodia. Elimination of Fascin resulted in reduced straightness of microspikes and a strong reduction of average filopodia formation frequency, although few filopodia were still observed. Interestingly, both Fascin and T-plastin KO clones displayed faster rates of random cell migration, suggesting an inverse correlation between migration speed and frequency of filopodia formation. Moreover, T-plastin/Fascin double KO cells displayed additive defects in filopodia density and morphology, indicating that both proteins have distinct or partially redundant functions in filopodia

formation. Importantly, ultrastructural characterization revealed double KOs to display major defects in the arrangement of actin filaments within microspikes and filopodia. Despite the significantly reduced numbers of filopodia and clear structural defects upon simultaneous depletion of Fascin and T-plastin, inhibition of the lamellipodial regulator Arp2/3 complex in these double KO cells partially rescued filopodia formation. This suggests that competition between distinct actin machineries driving various types of protrusion (lamellipodia *vs* filopodia) may impact on the requirement of actin bundling proteins for filopodia formation.

P1831/B70

Differential Properties of Calponin-like Repeats Regulate Actin-bundling Activities of Calponin-related Proteins.

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Calponins and calponin-related proteins regulate actin filament bundling, actin filament stability, and actomyosin contractility in a wide variety of cell types. Many of these proteins contain multiple calponin-like (CLIK) repeats as actin-binding sites. Previous studies have shown that the presence of multiple CLIK repeats in these proteins confers strong actin-bundling activities. However, whether the CLIK repeats play similar redundant roles or have distinct functions remains unknown. Here, we investigated actin-bundling properties of UNC-87, a *Caenorhabditis elegans* calponin-related protein with seven CLIK repeats, and found evidence that differential properties of CLIK repeats are important for the actin-bundling activity of UNC-87. We found that binding of UNC-87 to actin filaments was resistant to high-salt (0.5 M potassium chloride) but actin-bundling by UNC-87 was significantly reduced under high-salt conditions. Under physiological conditions, UNC-87 bound to actin filaments in the presence of tropomyosin, but actin-bundling by UNC-87 was partially inhibited by tropomyosin. These results suggest that an actin-binding site of UNC-87, which is required for actin-bundling, is sensitive to high-salt and tropomyosin. CLIK-1 is another *C. elegans* calponin-related protein with seven CLIK repeats. CLIK-1 and UNC-87 share 42 % identity (56 % similarity) in their amino acid sequences. Interestingly, CLIK-1 bound to actin filaments without bundling filaments. CLIK-1 and UNC-87 competed for binding to actin filaments, and CLIK-1 partially inhibited actin-filament bundling. By comparing the CLIK repeats in CLIK-1 and UNC-87, the greatest difference was found in the most C-terminal CLIK repeats in the two proteins, suggesting that these are the determinants for the actin-bundling activity. In *C. elegans*, CLIK-1 was expressed in body wall muscle, gonadal myoepithelial sheath, and vulval muscle, where UNC-87 was also expressed. These results suggest that differential properties of CLIK repeats confer distinct actin-regulatory functions of the two calponin-related proteins in *C. elegans*.

P1832/B71

The Mechanical Properties of a Utrophin Construct Encoding the Tandem CH Actin Binding Domain through Spectrin Repeat 3 Is Altered by the Cell Expression System through Post-translational Modification.

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Duchenne muscular dystrophy (DMD) is a lethal muscle wasting disease caused by the absence of dystrophin protein. Utrophin is a dystrophin homologue currently under investigation as a replacement therapy for DMD. Dystrophin and utrophin are hypothesized to function as molecular shock absorbers to mechanically stabilize the muscle cell membrane. Recently, we published atomic force microscopy data showing that utrophin is much stiffer than data previously reported for dystrophin. Here we show that the cell expression system employed impacts both the post-translational modification and mechanical behavior of a utrophin construct encoding the N-terminal actin binding domain through spectrin repeat 3 (UtrN-R3). UtrN-R3 expressed in insect cells showed significantly higher unfolding forces than when expressed in bacteria. Other analyses showed phosphorylation of UtrN-R3 from insect cells but not bacterial UtrN-R3. Our results demonstrate that the mechanical properties of utrophin are affected by the cell expression system employed and phosphorylation status. Our experiments also suggest a potential mechanism by which the mechanical stiffness of utrophin (and possibly dystrophin) can be regulated in *vivo*.

P1833/B72

β_H-spectrin Recruits PP2A to Crumbs to Regulate Crosstalk with the Hippo/Warts Pathway in *Drosophila*.

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Spectrin is a large F-actin crosslinking protein that most famously forms 2D networks in association with the plasma membrane of red blood cells. There it confers cell shape and strength during the rigors of circulation. In NON-erythroid tissues, spectrin has additional roles in the endomembrane system. We have previously shown that the apically-polarized β_H-spectrin, encoded by the *karst* locus in *Drosophila*, is required for the stability of several apical proteins, through the promotion of endosomal recycling to the plasma membrane - so called, 'dynamic protein stabilization'. The apical protein determinant Crumbs recruits β_H to the apical membrane and is itself trafficked in a β_H-dependent manner. β_H binds to the Hippo/Warts pathway (HWP) regulator Expanded, which mediates Crumbs crosstalk to the HWP. Here we report that a yeast 2-hybrid (Y2H) screen identified the PP2A substrate-specificity subunit Waldorf (a PP2A-PR72/B'' isoform) as a binding partner of β_H-spectrin. Waldorf binds to β_H *via* a short conserved sequence in the globular segment 33 of β_H. Genetic interaction and molecular epistasis experiments strongly suggest that PP2A with the Waldorf specificity-subunit bound to it ($PP2A^{Waldorf}$) acts as a negative regulator of Crumbs by acting to displace aPKC from Crumbs. Consistent with this notion, mutant versions of Crumbs lacking target residues for aPKC in the FERM-domain binding site do not respond to changes in Waldorf levels. The 'output' of Crumbs that appears to be most sensitive to the levels of Waldorf is crosstalk with the HWP and not apical polarity *per se*. Thus

knockdown/overexpression of Waldorf results in the under/overgrowth of wing tissue, and Waldorf has a genetic interaction with Yorkie, but apicobasal polarity is not lost. Waldorf also modulates protein trafficking in a similar way to β_H and its previously reported partner an nexin B9: Knockdown of Waldorf leads to an increase in Rab7-positive and acidic compartments, suggesting that $PP2A^{Waldorf}$ also normally acts by suppressing lysosomal trafficking, most likely in favour of recycling pathways. Our results support a model in which Crumbs recruits β_H in a complex with the HWP activator Expanded, and $PP2A^{Waldorf}$ bound to β_H acts in a homeostatic fashion to limit Crumbs activation of the HWP by displacing aPKC complex. This in turn limits the amount of growth suppression caused by Crumbs-dependent HWP activation.

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Regulation of Actin Dynamics 3

P1834/B73

Actin Bundle Assembly by Formins and Mechanics.

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Formins are central players in the assembly of most actin networks in cells, but how their activity is affected by the geometrical constraints imposed by the network architectures, such as filament crosslinking and formin spatial confinement, is still unclear. We have combined microfluidics and micropatterning techniques to investigate in vitro the dynamics of actin filament bundles induced by fascin and elongated by mDia1 formins. We have measured the impact of geometrical constraints on formin elongation rates and processivity. We show that filament bundling by fascin reduces formin activity. Strikingly, when formins are surface-anchored and elongate cross-linked filaments, formin elongation rate immediately decreases and processivity is greatly reduced, depending on the cumulative impact of formin rotational and translational freedoms, revealing an unexpected crosstalk between the constraints at the filament and the formin levels. Those results highlights the importance of molecular details of the formin anchoring to the plasma membrane in cells to efficiently modulate formin activity.

P1835/B74

The Formin Inhibitor, Smifh2, Inhibits Members of the Myosin Superfamily.

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The formin inhibitor, SMIFH2, is widely used in cell biological studies. It inhibits formin-driven actin polymerization in *vitro* with half maximal inhibition (IC50) values ranging from 5 to 15 µM for different formins. In search of formin function in F-actin retrograde flow, we examined the effect of SMIFH2 on the centripetal movement of actomyosin transverse arcs in detergent-permeabilized cells induced by ATP addition. In this system, neither polymerization nor depolymerization of actin can occur, because of the absence of G-actin and the presence of F-actin stabilizer, phalloidin. Nevertheless, SMIFH2 strongly inhibited or completely abolished the centripetal movement of transverse arcs in this system, as well as ATP-dependent contraction of linear stress-fibers. This caused us to question whether SMIFH2 might also be inhibiting non-muscle myosin 2A. Surprisingly we found that SMIFH2 inhibited both the actin activated MgATPase activity (IC50 50 µM) and the rate of actin filament sliding in the in *vitro* motility assay of this myosin (no movement detected at 150 µM SMIFH2). Interestingly, the effect of SMIFH2 on

motility could not be reversed by washout of the flow cell. We next tested the effect of SMIFH2 on other myosin family members. The ATPase activity of skeletal muscle myosin 2 was inhibited with an IC50 of 40 µM. Similarly, the ATPase activity of myosin 10, which is involved in the formation of filopodia in mammalian cells, was inhibited by SMIFH2 with an IC50 of 15 µM. Moreover, ATPase activity of *Drosophila* myosin 5 was inhibited much more potently with an IC50 of 2µM, that is even more potently than SMIFH2 inhibits most formins. Based on this information, we recommend that this inhibitor not be used in cell-based assays where myosins are always present.

P1836/B75

Mechanical Force Promotes the Dissociation of Arp2/3 Complex Branches.

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The branched actin network, primarily comprised of actin and the Arp2/3 complex, generates and sustains force to power cellular functions like motility, endocytosis, and vesicle trafficking. During assembly of the branched actin network, it experiences variable resistance from the cell membrane and responds by adapting it's growth speed, power, and architecture both in vitro and in cells. The network also experiences mechanical force during disassembly but much less is known about how forces affect the disassembly of actin filament networks. Here, we examine how mechanical forces affect disassembly of branched actin networks and focus on the dissociation rate of Arp2/3 complex branches. We used microfluidics to apply force to branches formed from purified actin and Arp2/3 complex and observed debranching events in real time with TIRF microscopy. We found that low pN forces on branches dramatically accelerated the dissociation of branches from the mother filament. We find that the Arp2/3 complex exists in distinct mechanical states and each mechanical state has different sensitivity to force. The mechanical states and sensitivity to force is regulated by the ATPase cycle of the Arp2/3 complex. Interestingly, we also show that the ATPase cycle of the Arp2/3 complex also regulates it's interactions with debranching proteins such as Glia maturation factor (GMF). These observations suggest that the ATPase cycle of the Arp2/3 complex tunes sensitivity to debranching by force and regulatory proteins. These findings have broad implications for cell biology because they reveal a mechanism were older portions of the branched actin network could be specifically targeted for disassembly by force and other debranching proteins.

P1837/B76

Twinfilin Uncaps Actin Filament Barbed Ends to Promote Turnover of Lamellipodial Actin Networks.

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Coordinated polymerization of actin filaments at their barbed ends towards membrane generates pushing force, which is essential for leading edge protrusions during cell migration and membrane invagination in endocytosis. Among the most central regulators of actin filament polymerization is the heterodimeric capping protein (CP). It binds to filament barbed ends with high affinity and slow dissociation kinetics to prevent their polymerization and depolymerization. In cells, however, CP displays very dynamic association with actin filaments, but the underlying mechanism has remained elusive. Here we reveal that another ubiquitous cytoskeletal regulator, twinfilin, which interacts with CP, actin

monomers and filament barbed ends, is a critical regulator of CP dynamics in vitro and in cells. By generating twinfilin-1/twinfilin-2 knockout B16-F1 cells, we learned that the lamellipodial actin filaments are less dynamic in the absence of twinfilins, and that this leads to diminished protrusion velocity. With in *vitro* single filament imaging coupled with microfluidics, we revealed that twinfilin does not accelerate actin filament barbed end depolymerization. Instead, these experiments demonstrated that twinfilin drastically accelerates CP dissociation from filament barbed ends through a mechanism that is dependent on twinfilin's actin-binding ADF-H domains. Consistently, loss of twinfilins in B16-F1 cells resulted in ~10-fold slower dynamics of CP, and this could be rescued with expression of wild-type twinfilin-1 but not by a twinfilin-1 mutant in which the ADF-H domains were inactivated. Moreover, whereas in wild-type cells CP was enriched at the distal edge of lamellipodial actin filament networks, it localized throughout the entire lamellipodium in twinfilin-deficient cells. Together, these results uncover the cellular function of twinfilin, and demonstrate that rapid dynamics of CP in cells, as well as its specific accumulation to the very leading edge of lamellipodium, are dependent on filament uncapping by twinfilin.

P1838/B77

Mechanical and Biochemical Factors Regulating the Disassembly of Actin Filaments.

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The disassembly of actin filaments is essential and must be tightly regulated in cells. The central player in actin filament disassembly is the ADF/cofilin protein family. Understanding their action on actin filaments is challenging because it comprises different reactions, including: binding to filaments, severing filaments, and affecting the monomer off-rate at both ends. In addition, actin turnover rates can vary greatly, as the action of ADF/cofilin is modulated by other proteins, by post-translational modifications, by ionic strength and pH, and by mechanical factors. Using microfluidics to study single actin filaments, we quantify the key reactions of ADF/cofilin, independently and in different contexts. In particular, we show that, quite unexpectedly, ADF/cofilin can drive the depolymerization of filaments from the barbed end in physiological conditions. Moreover, we show that the mechanical context of the filaments, and in particular, torsional constraints, can greatly enhance cofilin-induced severing. Our results illustrate the emerging notion that a variety of factors, very different in nature, co-regulate cytoskeletal dynamics.

P1839/B78

Arp3B Containing Arp2/3 Complexes Promote Disassembly of Branched Actin Networks.

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Branched actin networks generated by the Arp2/3 complex are essential for many different cellular processes, including cell motility, endocytosis and phagocytosis. In addition, a number of intracellular pathogens such as Listeria, Shigella and Vaccinia virus recruit and use the power of Arp2/3 driven actin polymerization to enhance their cell-to-cell spread. The Arp2/3 complex is composed of seven subunits (Arp2, Arp3, ARPC1-5). Interestingly, in humans, Arp3, ARPC1 and ARPC5 exist as two different isoforms (Arp3/Arp3B, ARPC1A/ARPC1B and ARPC5/ARPC5L) that are 91, 67 and 67% identical respectively. This raises the possibility that Arp2/3 is actually a family of 8 complexes with different interactions and

properties. Using Arp2/3 driven motility of Vaccinia as a model system, we have previously demonstrated that ARPC1 and ARPC5 isoforms confer different actin nucleating properties to the Arp2/3 complex (Abella et al., 2016). Our unpublished data now demonstrates that Arp3 and Arp3B also impart different properties to the Arp2/3 complex, despite the proteins being 91% identical. Depletion of Arp3B in HeLa cells results in increased stability of Arp2/3 branched actin networks and faster actin-based motility of Vaccinia. In contrast, over-expression of Arp3B suppresses Arp2/3-driven actin polymerization. In *vitro* assays with recombinant proteins, however, demonstrate that Arp3 and Arp3B containing complexes are equally efficient at assembling actin filaments. Therefore, we examined whether Arp3 and Arp3B impact instead on the stability of the branched actin networks that they generate. Using photoactivatable Arp3 and Arp3B constructs, we find that Arp3B containing-complexes have a significantly faster turnover in branched actin networks. Our collective observations are consistent with a model in which Arp3B containing Arp2/3 complexes promote disassembly of branched actin networks. By analysing a series of Arp3:Arp3B hybrids, we have identified the residues which likely contribute to these differences. Taking into account the ARPC1 and ARPC5 isoforms, our study now clearly demonstrates that in humans the Arp2/3 complex is indeed a family of 8 different complexes, each with unique properties.

P1840/B79

Single-molecule Imaging Reveals Dual Actin Regulatory Roles for IQGAP1.

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IQGAP1 is a key effector of Rac1 and Cdc42 that binds to F-actin and links cell signaling events to cytoskeletal rearrangements. Although IQGAP1 is conserved from yeast to humans, and is critical for many actin-based processes including cell motility and cytokinesis, the nature of its interactions with actin filaments and its effects on actin dynamics and spatial organization have remained somewhat elusive. Here, we show that full-length human IQGAP1 forms dimers, which by electron microscopy have a large globular domain (formed by its C-terminal halves) with two long extensions (formed by its N-terminal halves). Using in *vitro* single-molecule imaging, we show that IQGAP1 molecules interact tightly with the sides of filaments (dwell time > 15 min) and organizes them into thin bundles. These activities depend on the N-terminal half of IQGAP1. In contrast, the C-terminal half of IQGAP1 binds transiently to the barbed end of the filament (dwell time ~ 30 sec) and pauses filament growth while it is bound. Decoration of filaments with IQGAP1 also dramatically slows filament disassembly. Thus, IQGAP1 has dual functions as a transient barbed end capper and a filament bundling and stabilizing protein, likely to underlie many of its previously described in *vivo* roles.

P1841/B80

Investigating the Spatiotemporal Regulation of Arp2/3 and Branched Actin Networks during Cell Migration.

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Cell migration is essential for many important physiological processes, including embryonic development, wound healing, and the immune response. In addition, the signaling pathways and mechanisms governing cell motility are often aberrantly utilized to facilitate cancer metastases. Cell migration can be directionally biased by extracellular guidance cues. The sensing of gradients of

substrate-bound cues through integrin receptors, known as haptotaxis, requires the highly conserved Arp2/3 complex and its critical role in the polymerization of branched actin networks and the generation of lamellipodial cellular protrusions. Using highly tractable embryonic fibroblast cell lines derived from an *Arpc2* conditional knockout mouse, we seek to explore novel aspects of how Arp2/3 and branched actin are regulated by diverse extracellular substrates. CRISPR-mediated labeling of the essential Arpc2 subunit of the Arp2/3 complex allows us to observe branched actin dynamics and affords optogenetic control of the endogenous Arp2/3 complex. High-resolution time-lapse imaging reveals cells plated on fibronectin-coated, integrin engaging substrates have striking differences in the organization and behavior of Arp2/3 and actin-based structures when compared to cells plated on Poly-L-Lysine, which lack substrate-bound integin signaling ligands. In the absence of integrin engagement, optogenetically sequestering Arp2/3 to the mitochondria reveals protrusions in this context are being primarily supported by linear actin bundles, with a minor contribution of Arp2/3 and branched actin, but the release of Arp2/3 from sequestration shows that simply transiently increasing the local concentration of Arp2/3 in protrusions is sufficient to push the balance more towards branched actin polymerization. Micropatterning experiments reveal that Arp2/3 is enriched in protrusions as they push across fibronectin islands and that mere close promixity to fibronectin engagement is not sufficient for this enrichment. Genetic or pharmacological perturbation of several components required for haptotaxis can alter Arp2/3 and protrusion dynamics in cells plated on fibronectin, but none as of yet have been found to mimic the effects of cells plated sans integrin ligand. Additional steps are being taken to further characterize this intimate relationship between integrin-based adhesion signaling and Arp2/3 regulation. Ultimately, these efforts are geared towards developing a deeper and more comprehensive understanding of fundamental cell behaviors by examining how the motility machinery is assembled and organized within cells in response to extracellular cues.

P1842/B81

Assembly of Divergent Actin Rings in *Chlamydomonas*Is Both Formin and Arp2/3 Dependent but Myosin Independent.

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Chlamydomonas reinhardtii, a biflagellate green alga, serves as an important model for understanding cilia, photosynthesis, and increasingly actin regulation due to the co-expression of two actins with ~63% sequence identity. *Chlamydomonas* has a conventional actin gene, IDA5, as well as an unconventional actin gene, NAP1. Our previous studies have demonstrated how treatment of wild type cells with latrunculin B (Lat B), an actin depolymerizing agent, results in the formation of a perinuclear ring. We previously showed that NAP1, which is strongly upregulated upon Lat B treatment and insensitive to Lat B, is both necessary and sufficient for formation of these phalloidin-labeled structures. Optimizing the conditions for high incidence of rings demonstrates the importance of strain background, LatB treatment time, light conditions during growth and treatment, and cell culture density. To determine which proteins are important for NAP1 ring formation and maintenance, we looked first at myosin. Treating wild type cells with both LatB and blebbistatin, a myosin inhibitor we previously showed could recapitulate specific actin-dependent phenotypes in *Chlamydomonas*, resulted in no significant difference in the percentage of cells with rings. Conversely, inducing rings in the presence of a formin inhibitor, SMIFH2 completely blocked initial ring formation but didn't affect integrity of already-formed rings. Insertional mutant for the formin FOR1 also produced no rings, confirming the requirement for this actin nucleators in ring assembly. Additional data from the lab shows similar results for Arp2/3, a

branched actin nucleator. Due to the requirement for both nucleators for producing a filamentous actin structure comprised of NAP1 suggests FOR1 and Arp2/3 may be able to nucleate filaments comprised of this divergent actin.

P1843/B82

Investigating the Regulatory Role of the Extracellular Matrix in Cortical Brain Development.

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Changes in the ratio of excitatory to inhibitory (E/I) synapses is a common underlying mechanism of many neurological diseases. Increased hyperexcitability of cortical neurons is characteristic of neurodevelopmental disorders, such as epilepsy and autism spectrum disorders. Currently, we know little about how this E/I ratio is established. However, changes in the extracellular matrix (ECM) during neurodevelopment may alter synapse formation. Hyaluronan (HA), the major component of the brain ECM, is a macromolecule that controls cellular spacing. Through interaction with its receptor, CD44, HA regulates RhoGTPase signaling pathways. RhoGTPases are master regulators of actin organization. Since the actin cytoskeleton is enriched in excitatory synapses, we hypothesize that HA restricts excitatory synapse formation through regulation of RhoGTPase signaling. Using human-derived cortical brain spheroids, we manipulated HA levels and observed the resulting effects on synapse formation and neurotransmission. Consistent with our hypothesis, enzymatic digestion of hyaluronan leads to increased excitatory synapses and decreased inhibitory synapses. The elevated excitatory synapse formation resulted in increased spontaneous neural activity. In contrast, the addition of high molecular weight hyaluronan into the environment has opposite effects, decreasing excitability and resulting in decreased spontaneous neural activity. Protein analysis of RhoGTPase activity confirms these results. These data support a regulatory role for the ECM in cytoskeleton remodeling at the synapse and establish a new model of ECM regulation of E/I imbalances associated with human neurodevelopmental disorders.

P1844/B83

Characterization of CARMIL-GAP, a *Dictyostelium* CARMIL Isoform Harboring a GTPase Activating Domain for Rac.

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CARMILs (Capping protein Arp2/3 Myosin I Linker) are ~1000 residue, multi-domain scaffold proteins expressed from protozoa to man that have been studied extensively with regard to their ability to bind Capping Protein (CP) and reduce its affinity for the actin filament barbed end. CARMIL proteins also appear to play important roles in signal transduction, as they exhibit genetic and physical interactions with the Rac GEF Trio (Liang et al MBoC 2009; Vanderzalm et al. Dev. 2009), and T cells lacking CARMIL-2 exhibit a profound block in signaling downstream of CD28, the major co-receptor for T cell signaling (Liang et al Nat. Immunol. 2013). In previous work (Jung et al JCB 2001), we showed that Dictyostelium CARMIL binds CP, the Arp2/3 complex and myosin I (through its SH3 domain), and it is required for actin-dependent processes such as chemotaxis and micropinocytosis to be robust. Here we describe initial studies of CARMIL-GAP, a second Dictyostelium CARMIL that contains, in addition to all the normal CARMIL domains (including the CP-binding CPI domain), a ~130 residue insertion that, by homology, is a

GTPase activating (GAP) domain for Rho-GTPases. This domain is probably functional given that full length CARMIL-GAP can only be over-expressed if its GAP domain contains a point mutation (R737A) that blocks GAP activity in all characterized GAP proteins. Moreover, MS analyses of GAP domain pull-downs indicate that CARMIL-GAP binds the Rac isoform Rac1A. Like CARMIL, CARMIL-GAP localizes to actin-rich structures and is expressed in both vegetative and starved, developing cells. Consistently, CARMIL-GAP null cell lines created by homologous recombination exhibit pronounced defects in several actin-based processes occurring in vegetative and starved cells, including phagocytosis, motility and chemotactic aggregation. Importantly, these defects are specific, as expression of GFP-FL CARMIL-GAP in CARMIL-GAP null cells rescues them. Finally, rescue of CARMIL-GAP null cells with versions of CARMIL-GAP that lack either GAP activity or the ability to bind CP show that while both domains contribute significantly to CARMIL-GAP function, the GAP domain plays the bigger role.

P1845/B84

Spire1C and the Arp2/3 Complex Drive a Wave of Filamentous Actin That Promotes Mitochondrial Fission and Motility.

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Mitochondria are dynamic organelles that undergo fission and fusion and move throughout the cell. The main driver of organelle dynamics is the cytoskeleton. While it is well established that mitochondria are trafficked along microtubules, interactions between mitochondria and actin are less well understood. Novel results from our lab expand on the role of actin in mitochondrial dynamics. Using spinning disk and lattice light-sheet microscopy our lab identified in both immortalized and primary cell types a wave of filamentous (F-) actin that propagates through the mitochondrial network cyclically assembling and disassembling on adjacent subpopulations of mitochondria. In interphase the F-actin wave promotes mitochondrial fission; once actin disassembles daughter organelles often fuse with distinct neighbors leading to mitochondrial mixing. During metaphase the F-actin wave promotes mitochondrial motility via the formation of actin comet tails. To investigate the mechanism underlying the F-actin wave we used both pharmacological inhibitors and RNAi and determined that the wave depends on a diverse set of proteins that facilitate F-actin assembly and disassembly. Specifically, the F-actin wave is regulated by CDC42 and requires the Arp2/3 complex, VASP, and Spire1C. Actin filaments within the wave recruit the stabilizing factor, Filamin A. Active depolymerization of F-actin is also required to maintain the integrity of the wave as siRNA-mediated depletion of ADF, Gelsolin, or Cofilin resulted in significant enlargement of wave size. We propose that the function of the F-actin wave is to allow spatial mixing of mitochondrial contents, including mitochondrial DNA.

P1846/B85

Identification of a Novel Regulatory Circuit That Modulates the Actin Cytoskeleton during Sea Star Meiosis.

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Meiosis in oocytes represents a highly atypical cell division that results in a single viable gamete. In the sea star, oocytes are arrested in G2 of meiosis I, and upon stimulation with the 1-methyladenine (1-MeAde), a signal transduction pathway involving heterotrimeric G proteins and phosphatidylinositol-3

kinase (PI3K) stimulates M phase entry within 30 minutes. To better understand the regulation of the actin cytoskeleton during meiotic maturation, we performed a combination of live cell imaging and micromanipulation on *Patiria miniata* oocytes prior to- and following hormone activation. 4D imaging of oocytes expressing GFP-Lifeact or a biosensor for RhoA activity revealed a transient elaboration of actin-based projections and a general cortical enrichment approximately 2 minutes post-stimulation that decreased to below pre-hormone levels by Germinal Vesicle Breakdown (GVBD). Measurements of cortical tension mirrored these dynamics, with initial tension levels being high prior to hormone activation and dropping dramatically by GVBD. Inhibition of RhoA, either with dominant-negative Rho or a ROCK inhibitor depressed the cortical actin response as well as overall cortical tension levels, suggesting that Rho-ROCK signaling was a key regulator of cortical actin during this period. Inhibition of CDK activity had no effect on the decrease in cortical tension, suggesting that the drop in Rho activity and cortical tension was not a function of M phase entry. In contrast, inhibition of PI3K activity blocked GVBD and entry into M phase, but Rho activity and cortical tension remained elevated, suggesting that PI3K may play a feedback role in downregulating Rho. Together, these data suggest that hormone activation not only triggers meiotic re-entry, but also modulates Rho family GTPases to effect changes in the cortical actin cytoskeleton to promote cortical maturation in preparation for fertilization and development.

P1847/B86

Balanced Rho Activation and Inhibition Regulates Exocytosis by Large Secretory Vesicles.

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The small GTPase Rho governs actomyosin-based contractility in a variety of cellular settings, by the parallel induction of actin polymerization and of myosin II recruitment and activation. Regulation of Rho function is mediated by guanine exchange factors (GEFs) and GTPase activating proteins (GAPs), which stimulate and inhibit Rho activity, respectively. We have been exploring the dynamics and molecular design of this fundamental circuitry in the context of exocytosis. Specifically, we study secretion of adhesive “glue” proteins (mucins) from the epithelium of the *Drosophila* salivary gland as a model system. Glue protein exocytosis is achieved via uncommonly large secretory vesicles (>5 um in diameter). Rho-mediated assembly and contraction of an actomyosin coat that forms around these vesicles upon their fusion with the apical cell membrane, is critical for release of the glue material into the gland lumen. We have identified RhoGEF2 as the activator of Rho in this setting. RhoGEF2 is recruited to the fused vesicles, and its function is essential for activation of Rho (as monitored with an active Rho sensor) on the vesicle surface, and for vesicle contraction. Interestingly, an actomyosin coat still forms around the vesicles following knockdown of *RhoGEF2*, likely generated by basal levels of Rho-GTP, which diffuse from the apical membrane following fusion. RhoGEF2 recruitment is actin dependent, implying an amplification mechanism for establishing a coat sufficiently robust to enable contraction. This process is counteracted by a dedicated RhoGAP, RhoGAP71E, which is also recruited by actin, and whose inhibitory function is essential for vesicle contraction. Content release from the giant secretory vesicles thus appears to require a finely timed balance between Rho activation and inhibition. A major challenge is to determine how temporal order is maintained within this circuitry, particularly since actin appears to serve as a shared recruiting element of both activating and inhibiting factors. A corresponding effort aims to elucidate the mechanisms underlying the recruitment and contractile

activity of myosin II, which is distributed in a non-homogeneous, “stripe”-like pattern on the vesicle surface.

P1848/B87

Centrocortin Regulates Axial Nuclear Migration in a Rho1-dependent Manner in the Early *Drosophila* Embryo.

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In the early *Drosophila* embryo, the first 14 nuclear divisions occur rapidly and synchronously in a syncytium absent of cellular membranes. After the fourth nuclear division, the nuclei spread out along the anterior-posterior (A-P) axis of the embryo to prepare for future patterning steps during a process termed axial nuclear migration. This nuclear spreading relies on actomyosin contractions localized at the cortical plasma membrane that regulate cytoplasmic streaming, which subsequently distributes the nuclei along the A-P axis. Axial nuclear migration is critical for positioning the nuclei at the posterior pole in the proper time window for gametic cell differentiation, as well as regulating the subsequent symmetric nuclear divisions. Axial nuclear migration is necessary for successful development, yet the molecular mechanisms and pathway components that regulate it remain unclear. Our investigations reveal two important components that cooperate to regulate axial nuclear migration: the conserved centrosomal actin regulatory protein Centrocortin (Cen), and one of the major GTPases involved in actin regulation, Rho1. Combining hemizygous *cen* mutants with heterozygous *rho1* mutants (*cen* -/-; *rho1* +/-) results in severe axial nuclear migration failure that is not present in the single mutants, indicative of a cooperative role. The defects in axial nuclear migration result in reduced embryo survival and sterile offspring that are devoid of a germline due to defective pole cell formation. Overexpression of Cen results in asynchronous nuclear divisions, however, axial nuclear migration remains intact. We also found that Cen interacts physically with multiple actin regulators such as Filamin, Formin, and other actin-associated proteins through mass spectrometry analysis. A genetic screen using RNAi lines of actin and myosin regulators in the early embryo in combination with *cen* RNAi identified interactions of *cen* with other components in the Rho1 pathway. Altogether, these data show that Cen is a novel actin regulator that cooperates with Rho1 to regulate the actomyosin dynamics necessary for axial nuclear migration in the early *Drosophila* embryo. In this study, we aim to elucidate the role of Cen in the early embryo as an actin regulatory protein and establish its position in the Rho1-mediated pathway that regulates axial nuclear migration.

P1849/B88

Lipid Droplets Contribute to Actin Cytoskeletal Maintenance during *Drosophila* Oogenesis.

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Lipid droplets (LDs) are ubiquitous cellular fat storage organelles with critical roles in energy homeostasis and metabolism. In addition, LDs also function in protein storage and defense against pathogens. Here, we uncover a novel role for LDs in maintaining the actin cytoskeleton during *Drosophila* oogenesis. During oogenesis, the oocyte and its 15 sisters, the nurse cells, frequently remodel their actin cytoskeleton, leading to dramatic morphological changes. Exquisite temporal and spatial control of actin is in part mediated by prostaglandins (PGs), short-lived lipid signaling molecules synthesized by the COX-like enzyme Pxt. During mid-oogenesis, the cortical actin cytoskeleton in *pxt*

mutants breaks down, resulting in abnormal oocyte development and female sterility. We find that mutants in two LD proteins, the triglyceride lipase ATGL and the perilipin PLIN2, show similar cortical actin breakdown, even though Pxt protein abundance is normal. *ATGL* mutants genetically interact with *pxt* mutants, suggesting that ATGL and Pxt work in the same genetic pathway. We detect no enrichment of Pxt on LDs: biochemically purified LDs show no enrichment of Pxt, and Pxt localizes with either Golgi or ER markers, but not LDs, in *vivo*. These data suggest that Pxt does not act directly at the surface of LDs. Our working model is that the fatty acids used by Pxt to synthesize PGs originate from LDs and are released from the stored triglycerides in LDs by ATGL; PLIN2 may control ATGL activity. Both ATGL and PLIN2 are expressed during oogenesis and germline RNAi reveals that PLIN2, just like Pxt, is required in the germline for actin maintenance; similar experiments for ATGL are in progress. We also employed *DGAT1* mutants, in which nurse cells fail to make LDs. Mutant nurse cells eventually die, but display actin breakdown even before death, consistent with a role for LDs in actin maintenance. Using *in-vitro* culturing, it was previously shown that exogenous PGs can rescue the actin defects in *pxt* nurse cells. We are currently testing whether similar rescue is observed in *ATGL* or *PLIN2* mutants supplied with PGs or fatty acids. Our findings highlight unexpected, non-metabolic roles for LDs and LD proteins and link LDs to actin cytoskeletal regulation.

P1850/B89

Determining the Role for the Arp2/3 Complex and the Formin, Diaphanous, in the Germline of the Developing *Drosophila* Egg Chamber.

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Proper gamete formation is necessary for fertility in sexually reproducing organisms, and intercellular bridges are an essential structure found in the developing sperm and eggs from insects to mammals. The largest and most studied intercellular bridges are found in the developing fruit fly egg chamber. These intercellular bridges, or ring canals, connect the developing oocyte to supporting nurse cells. The ring canals allow neighboring cells to exchange material such as RNA, protein, and organelles, and defects in their stability or expansion lead to infertility. The ring canals are enriched in actin and actin binding proteins, and ring canal expansion depends on the activity of the Arp2/3 complex, which nucleates the formation of branched actin filaments. Mutations in members of the Arp2/3 complex lead to smaller and collapsed ring canals in mid-oogenesis, but it is not known whether the Arp2/3 complex plays an earlier role in oogenesis, or whether another actin nucleator, the formin, Diaphanous, is also required for ring canal formation or expansion. In this study, we use the GAL4/UAS system to study the effect of depletion of either the Arp2/3 complex member, ArpC2, or the formin, Diaphanous (Dia), from the developing germline. Our results suggest that Dia is essential for incomplete cytokinesis during germ cell divisions, and that both Dia and the Arp2/3 complex are required to regulate ring canal size throughout oogenesis. Additional studies are required to determine how the activity of these two actin nucleators is regulated as well as whether they may function antagonistically or cooperatively to regulate ring canal size.

Higher-Order Actin-Based Structures

P1851/B90

Coupling of Muscle Stress Fibers to the Extracellular Matrix Drives Their Maturation into Myofibrils in Cardiomyocytes.

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Forces generated by heart muscle contraction must be balanced by adhesion to the extracellular matrix (ECM) and to other cells for proper heart function. Loss of this force balance is thought to underly the progression of heart diseases such as dilated cardiomyopathies. Despite its clinical significance, how cell adhesion regulates the proper formation and maintenance of sarcomeres is poorly understood. A recent study proposed a new model of "centripetal assembly" in which sarcomeres arise *de novo* from sites of ECM attachment, i.e., focal adhesions. This group made this claim while imaging iPSC derived cardiomyocytes 24-48 hours after they were plated, a time window when sarcomeres are already assembled on the dorsal surface of the cell as we have recently shown. Since this group only imaged the ventral surface of the cells, their data seemingly shows sarcomeres appearing next to focal adhesions. However, close examination of this data revealed this group documented pre-formed sarcomeres at the dorsal surface coming into focus, which we experimentally verified. Instead of appearing out of adhesions, we found that *de novo* sarcomere assembly occurs by a completely different mechanism. Specifically, a population of stress fibers, termed muscle stress fibers (MSFs), undergo translocation away from the edge and transition into sarcomere containing myofibrils on the dorsal surface. We therefore tested the role of focal adhesions in regulating this transition. Live imaging of MSFs and focal adhesions showed that MSFs were connected to focal adhesions sporadically along their length through dorsal stress fiber-like connections during their transition to myofibrils. Furthermore, focal adhesions underwent maturation in parallel with sarcomere formation, suggesting that ECM adhesion may serve as a "clutch" to allow MSFs to generate more force, thus facilitating their maturation. In agreement with this hypothesis, ECM adhesion negatively correlated with the rate of translocation of MSFs away from the edge. Reducing cell ECM adhesion by reducing integrin ligand concentration, knockdown of focal adhesion kinase or over-expression of the head domain of talin led to attenuation of myofibril maturation and faster translocation of MSFs away from the edge. Conversely, increasing ECM adhesion by inhibition of the auto-phosphorylation of FAK or increasing integrin ligand concentration led to faster maturation of myofibrils and slower translocation of MSFs. These findings have led us to propose a counter-model for the role of cell-ECM during sarcomere formation to the one recently proposed; our model is similar in design principles to actin networks of crawling mesenchymal cells.

P1852/B91

Tropomodulins Stabilize Actin-tropomyosin Filaments in Migrating Cells to Control the Balance between Protrusive and Contractile Structures.

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Tropomodulins stabilize actin-tropomyosin filaments in migrating cells to control the balance between protrusive and contractile structures the actin cytoskeleton is important for a wide array of cellular and developmental processes, as well as for tissue homeostasis. To contribute to these diverse functions,

cells harbour different protrusive and contractile actin filament structures, which compete with one another for a limited pool of actin monomers. In order to regulate the dynamics of actin filaments, various actin-binding proteins have evolved. These include Tropomodulins (Tmods), which are actin filament pointed-end capping proteins. In muscle myofibrils, Tmods prevent the overgrowth of actin filaments, whereas their functions in non-muscle cells are less well understood. We show that, in U2OS osteosarcoma cells, Tmod1 and Tmod3 are integral components of actin stress fibers, and that they compensate for one another when depleted individually. However, simultaneous depletion of both the isoforms led to the disassembly of tropomyosin-actin filaments, lack of force-generating contractile stress fibres, and an increase in the cortical actin filament networks. Knockout-rescue experiments revealed that Tmod's ability to directly bind tropomyosin is important for stabilization of actin-tropomyosin filaments in cells. Moreover, by combining depletion of Tmods with an inhibition of the Arp2/3 complex, we provide evidence that loss of Tmods shifts the balance from formin-assembled actin filament structures to Arp2/3 complex-nucleated branched networks. This shift in balance between actin filament networks upon Tmod's depletion can be partially rescued by inhibiting the Arp2/3 complex or by depleting Arp2/3 by siRNA. Collectively, this study demonstrates that in non-muscle cells, Tmods are critical for the stability of tropomyosin-actin filaments, and thus for the maintenance of actin cytoskeleton homeostasis.

P1853/B92

Non-muscle Myosins 2 in Cell Polarization and Contact Guidance.

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Contact guidance refers to the ability of cells to sense the geometrical features of the microenvironment and respond by changing their shape and adapting the appropriate orientation. We have discovered that contact guidance is preserved following loss of nonmuscle myosin (NM) 2B and that expression of NM2A alone is sufficient to establish an appropriate orientation of the cells. Loss of NM2B, and overexpression of NM2A, results in prominent cell polarization that is found to be linked to the increased alignment of microtubules with the actomyosin scaffold. Lack of mechanosensitive NM2B in stable stress fibers affects maturation of the focal adhesions as well as maturation of the actomyosin cytoskeleton. Thus for spreading cells, the loss of NM2B results in numerous thin and dynamic stress fibers which form tightly packed arrays at the cell-surface interface due to the formation of large numbers of small immature focal adhesions at the leading edge. Consequently, the spreading history of cells, encoded by stress fiber network organization, affects organization of microtubules that induce and maintain cell polarity by their structural adaptation to the geometry of the actomyosin at the cell-surface interface.

P1854/B93

The Transition of Podosomes into the Zipper-like Structures in Macrophage-derived Multinucleated Giant Cells.

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Macrophage fusion resulting in the formation of multinucleated giant cells (MGCs) is a multistage process that requires many adhesion-dependent steps and involves the rearrangement of the actin

cytoskeleton. The diversity of actin-based structures and their role in fusing macrophages is poorly understood. In this study, we revealed the hitherto unrecognized actin-based zipper-like structures that form between MGCs generated on the surface of implanted biomaterials. We established an in *vitro* model for the induction of these structures in mouse macrophages undergoing IL-4-mediated fusion. Using this model, we show that the zipper-like structures develop mainly at cell-cell contacts between large MGCs and rarely between MGCs and mononucleated macrophages. Live cell imaging using macrophages isolated from mRFP- and GFP-Lifeact mice demonstrated that zipper-like structures are dynamic formations undergoing continuous assembly and disassembly and that podosomes are precursors of these structures. Immunostaining experiments showed that vinculin, talin, integrin $\alpha_M\beta_2$ and other typical components of podosomes are present in the zipper-like structures. Macrophages deficient in WASp and Cdc42, key molecules involved in actin core organization in podosomes, as well as cells treated with inhibitors of the Arp2/3 complex failed to form the zipper-like structures. Furthermore, E-cadherin and nectin-2 were found between adjoining membranes, suggesting that the transition of podosomes into the zipper-like structures is induced by bridging plasma membranes by junctional proteins.

P1855/B94

Mechanically Distinct Uptake Mechanisms of Antibody-mediated and Phosphatidylserine-mediated Phagocytosis Revealed by Microparticle Force Microscopy.

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Macrophages are able to phagocytose targets of vastly different rigidities, ranging from bacteria during immune response, to apoptotic cell fragments in tissue homeostasis. Phagocytosis can be strongly affected by minor differences in target rigidity and is generally less efficient for softer targets. How target rigidity is sensed by phagocytes, and how phagocytes adapt phagocytic mechanisms for efficient uptake of soft targets is currently poorly understood. Notably, soft targets like apoptotic cells are typically engaged through different ligands, such as phosphatidylserine (PtdS), than stiff targets such as bacteria, which are engaged through *e.g.* IgG. To reveal if uptake mechanics are ligand-dependent, we use microparticle traction force microscopy (MP-TFM), a methodology we recently developed to quantify the mechanical forces in immune-cell target interactions. First, monodisperse (CV < 0.1) deformable polyacrylamide microparticles with cell-like rigidities (Young's modulus 0.1 - 10 kPa) are synthesized using a membrane emulsification approach. These particles can readily be conjugated with a variety of ligands and fluorescent dyes. Using confocal microscopy, we can resolve the three-dimensional shape of individual particles during engulfment with 50 nm precision, after which we use a reference-free strategy to infer both shear and normal traction forces (>10 Pa) directly from the deformed particle shape. During IgG-mediated phagocytosis soft particles (0.3 kPa) are deformed strongly, implying highly localized cellular forces (~nN), and revealing 4 mechanically distinct steps in the phagocytic process. Initially, we observe outward directed pushing forces from the phagocytic cup base. During subsequent pseudopod extension the majority of the deformation is localized in a ring that is initially irregular, but becomes uniform during cup closure. Surprisingly, strong localized punches at the cup base still occur in these stages. After cup closure, we observe some of the strongest forces, seemingly pushing the engulfed target into the cell. These deformations correlate with local accumulation of actin and are likely intimately linked to cytoskeletal dynamics. In PtdS-mediated phagocytosis we observe a clearly distinct mechanical signature, with a striking overall elongation of the

particles and lower actin accumulation. Our novel approach gives us unprecedented detail on the mechanical interaction between phagocyte and target in phagocytosis and reveals critical differences between IgG- and PtdS-mediated phagocytosis.

P1856/B95

Phosphoinositides Regulate Force-independent Interactions between Talin, Vinculin, and Actin in Vitro.

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The focal adhesion (FA) proteins talin and vinculin connect integrin receptors in the membrane to contractile actomyosin networks, acting as the mechanosensitive core of FA complexes. Both proteins have been shown to directly bind F-actin, the phosphoinositide $PI(4,5)P_2$, and each other. Though these interactions are critical to FA formation, stability, and dynamics, the underlying mechanisms of regulation for talin and vinculin within FA complexes remain unclear. Here, talin-vinculin-actin assemblies have been reconstituted in vitro using various synthetic membrane systems, including liposomes, supported lipid bilayers, and encapsulation within giant unilamellar vesicles. Using biochemistry, light microscopy, and cryo-EM, we show that interactions between full-length talin and full-length vinculin are mediated by membrane binding. Previously, it has been suggested that force application is required for release of talin autoinhibition, leading to vinculin binding. Here, we show that the addition of phosphoinositide-rich membranes alone is sufficient to trigger talin-vinculin interactions, even in the absence of force. Additionally, membrane-bound talin and vinculin coordinate the recruitment and reorganization of F-actin at membrane surfaces, suggesting that local regulation of membrane composition could directly control the recruitment, activation, and engagement of proteins, such as talin and vinculin, within FAs.

P1857/B96

Generation of Stress Fibers from the Cortical Actomyosin Meshwork.

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Contractile actomyosin bundles are important for motile and morphogenetic processes in animal cells and tissues. Ventral stress fibers are the most prominent actomyosin structures in many non-muscle cell-types. These thick, contractile bundles are typically connected to focal adhesions at their both ends and contribute to cell migration, morphogenesis and mechanosensing. Ventral stress fibers can be generated either from the pre-existing network of two other types of actin bundles; transverse arcs and dorsal (radial) stress fibers (Hotulainen & Lappalainen, *J. Cell Biol.,* 2006; Tojkander et al., *Curr. Biol.,* 2011, Burnette et al., *Nat. Cell Biol.,* 2011; Tojkander et al., *eLife*, 2015; Fenix et al., *Mol. Biol. Cell.,* 2016; Hu et al., *Nat. Cell Biol.,* 2017), or via splitting of pre-existing ventral stress fibers (Young & Higgs, *Curr. Biol.,* 2018). In addition to thick ventral stress fibers, which are typically enriched at the lamellum of motile cells, many cell-types also exhibit thinner stress fibers at their posterior region (e.g. Burnette et al., *J. Cell Biol.,* 2014). Also these thin (basal) stress fibers are connected to focal adhesions at their both ends, but whether they are generated from the pre-existing network of transverse arcs and dorsal stress fibers, or through another mechanism, has remained elusive. Here, by utilizing 3D-SIM and live-cell TIRF microscopy on U2OS osteosarcoma cells and mouse embryonic fibroblasts (MEFs), we revealed that basal stress fibers assemble through NMII-dependent reorganization of the cortical actin meshwork at

the bottom of the cell. This leads to the formation of an actomyosin bundle, and subsequent enrichment of focal adhesion components at the ends of the bundle. The assembly of basal stress fibers is typically preceded by a NMIIA pulse at the ventral surface of cell, and appears to be dependent on formins but not the Arp2/3 complex. Basal stress fibers display periodic NMII pattern and are contractile, although based on traction-force microscopy they exert weaker forces to the substratum compared to thick ventral stress fibers. Live-cell imaging revealed that basal stress fibers undergo dynamic re-organization and are able to 'exchange neighbors': an event where the *de novo* forming actomyosin bundle interacts with another one and subsequently displaces its attachment from a focal adhesion to another in the near vicinity. Collectively, our work provides evidence that basal stress fibers are dynamic, contractile actomyosin bundles, which are generated through reorganization of the cortical actomyosin meshwork by a novel, NMII-dependent mechanism.

P1858/B97

Myofibril Assembly and the Role of the Ubiquitin Proteasome System.

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De novo assembly of myofibrils in vertebrate cross-striated muscles occurs in a step-wise formation that begins with **premyofibrils** built of thin actin filaments linked to muscle specific alpha-actinin. Small bands of nonmuscle myosin II filaments interact with actin filaments forming minisarcomeres. In a second step, incorporation of titin and muscle myosin II filaments onto premyofibrils leads to formation of **nascent myofibrils** with thin filaments of actin and two different isoforms of myosin II: nonmuscle myosin II in short filaments, and overlapping thick filaments of muscle myosin II. In the transition to **mature myofibrils,** late-assembling proteins (telethonin, myomesin, and muscle myosin II binding protein C) are inserted, nonmuscle myosin II is absent, and muscle myosin II filaments align into A-Bands with titin stretching from Z-Bands to the middle of the A-Bands. FRAP analyses of control myofibrillogenesis indicate that the dynamics of the assembling proteins decrease as premyofibrils transform to nascent myofibrils and then to mature myofibrils. In a search for controls of the process of myofibril assembly, we discovered that the transition from nascent to mature myofibril could be halted by inhibitors of the Ubiquitin Proteasome System (UPS). In particular, inhibition of E3 Cullin ligases halted the formation of mature myofibrils from nascent myofibrils. Inhibition of p97 extraction of ubiquitinated sarcomeric proteins also led to an inhibition of myofibrillogenesis at the nascent myofibril stage**.** We were able also to arrest myofibrillogenesis at the transition zone from nascent myofibrils to mature myofibrils by the use of five different proteasomal inhibitors. In contrast to the effects of various inhibitors of UPS, inhibitors of autophagy or lysosomes did not affect myofibrillogenesis. The similarity of the effects caused by three different classes of the UPS inhibitors indicates that removing and proteolyzing sarcomeric proteins from the nascent myofibrils is required to permit their transition to mature myofibrils. An alyses of the dynamics of three selected sarcomeric proteins (actin, muscle myosin II heavy chains and muscle myosin II light chains) by FRAP led to the discovery that the proteasome and the p97 inhibitors decreased the dynamics of each of these proteins in the first two stages of the three-step process of myofibril assembly, i.e. premyofibrils and nascent myofibrils, but not in the mature myofibrils. These results indicate that the exchanges of the sarcomeric proteins are important as premyofibrils and nascent myofibrils are transformed into mature myofibrils.

P1859/B98

Role of Amino Acid Sequence Differences in the Global Intracellular Functions of Beta- and Gamma-actin.

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Actin is one of the most essential and abundant eukaryotic proteins, highly conserved across the tree of life. Among the six mammalian actins, beta- and gamma-cytoplasmic actins are the only two that are ubiquitously expressed in every cell type and share the highest identity at the amino acid level, with only four homologous substitutions within their N-terminus. The underlying mechanisms maintaining their functional differences in vivo are unknown, and constitute one of the major unresolved questions in the field. Here we addressed this by using the mouse model recently developed in our lab, where beta-actin gene (Actb) is altered to encode gamma-actin protein from the endogenous Actb locus (Actbc-g mice). In these mice, beta-actin protein is absent, while the beta-actin gene is nearly intact, enabling us to address the direct effect of amino acid sequence on actin isoform function. While, consistent with previous reports, these mice are viable and appear phenotypically normal at the gross level, lack of beta-actin protein leads to specific defects in tissues that critically depend on the microvilli for their function, including the retina, and the brush border of the small intestine. Both tissues show major morphological changes in Actbc-g mice, including damaged microvilli of the retinal pigment epithelium that are responsible for photoreceptor homeostasis, as well as disorganization of the terminal web and altered microvillus length in the small intestine. In the retina, these defects are accompanied by progressive loss of light sensitivity. Biochemical studies link these defects to the altered binding of specific actin-associated proteins that exhibit a strong bias between beta- and gamma-actin. Our results suggest that beta-actin protein is essential for the microvilli maintenance in multiple cell types, and constitute the first demonstration of the key contribution of specific actin isoform's amino acid sequence to actin's in *vivo* functions. Overall, this study sheds major light on the functional differences between the two closely related cytoplasmic actins, which universally affect a specific subset of actin functions in multiple cell types.

P1860/B99

Barbed End-dependent Clustering of Vasp and Lamellipodin Initiates Filopodia within Lamellipodial Networks.

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The shape of many eukaryotic cells depends on interactions between the actin cytoskeleton and the plasma membrane. Changes in cell shape often result from protrusion of actin-filled pseudopods, including linear structures called filopodia. These filopodia can be created by a variety of mechanisms, including the dynamic reorganization of growing filaments within a two-dimensional lamellipod. A key protein required for the emergence of filopodia from lamellipodial networks is the actin polymerase VASP, whose clustering promotes the formation of linear actin bundles. By imaging the earliest events of this process in migrating B16F1 mouse melanoma cells, we discovered that nascent VASP clusters do not associate with the I-BAR-containing protein IRSp53, but arise from small, pre-existing clusters of the VASP-binding protein, Lamellipodin (Lpd). The small, Lpd-only clusters do not 'skate' across the leading edge the same way as VASP/Lpd clusters. Growth of VASP/lamellipodin clusters requires multivalent interaction between the two proteins as well as the presence of growing actin filaments. VASP/Lpd

clusters grow by accumulating monomers and by fusing with each other, but their size is limited by a previously undescribed, size-dependent instability.

P1861/B100

Chiral Bending of Filopodia.

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The mechanism underlying the left-right asymmetry in multicellular organisms is still poorly understood. Some evidence suggests that such asymmetry depends on the motile behaviour of individual cells. Indeed, different cell types, growing under isotropic conditions, demonstrate left-right chiral swirling of their actin cytoskeleton. This swirling depends on myosin-II driven actomyosin contractility and formin-mediated actin polymerization, and can be regulated by a number of actin-associated proteins [Tee et al, 2015]. The molecular events responsible for the swirling asymmetry, however, remain to be elucidated. Here, we show that filopodia growth in HeLaJW cells plated on the substrate covered by animal lectin (galectin-8) demonstrate local left-right asymmetry in their growth pattern. Specifically, those filopodia which bend during elongation do it in an asymmetric manner so that a fraction of “left-turning” filopodia was significantly higher than that of “right-turning”. Overexpression of myosin-X, a protein known to be involved in filopodia growth, significantly enhanced the fraction of bending filopodia (up to forty percent) on galectin-8-coated substrate. Over 80% of the ‘bending filopodia’ turned left. The asymmetry of filopodia turning was observed also in cells with inhibited myosin-II function and therefore depended mainly on actin polymerization and function of non-conventional myosins. Interference reflection microscopy (IRM) and TIRF microscopy showed that filopodia turning occurs only in filopodia with tips attached to the galectin-8-coated substrate. Moreover, the bending of filopodia was preceded by significant decrease in filopodia protrusion speed, suggesting that friction between filopodia tip and the substrate triggered the filopodia turning. Since filopodia growth is known to be driven by formin-dependent actin polymerization, we overexpressed the major formins, mDia1, mDia2, FMNL2, and DAAM1 in the cells, and found that the formin overexpressing cells produce less, yet longer and thicker filopodia as compared to controls. These filopodia, however, did not turn. An other method of stimulating filopodia formation and elongation is the knock-down of Arp2 or Arp3. We have found that filopodia induced by Arp2/3 depletion demonstrated myosin-X-dependent bending. This bending was also asymmetric with the preferential left turning, similarly to the filopodia induced by overexpression of myosin-X. Thus, integrin-independent filopodia adhesion to galectin-8 coated substrate revealed intrinsic asymmetry in actin-polymerization dependent and myosin-X -enhanced filopodia turning. Asymmetry of filopodia turning could be one of the factors determining left-right asymmetry in more complex multicellular systems.

P1862/B101

Reach Out and Touch Fate: Cytonemes in Sonic Hedgehog Signal Propagation.

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The formation and maintenance of morphogen gradients is essential during development, but the mechanisms by which morphogens are transported to form gradients are not yet clear. Evidence is mounting in support of their transport by long specialized filopodia known as cytonemes. Despite experimental evidence demonstrating cytoneme based transport of morphogens in vivo, insight into the

mechanisms of cytoneme initiation, regulation, and signal propagation is lacking. This is due to technical difficulties visualizing these fragile structures. We recently overcame this obstacle by developing a fixation technique termed MEM-fix that preserves the cytonemes of cells in culture, allowing investigation of fundamental questions about cytoneme biology, and the role they play in morphogen signal propagation. We find that cytonemes are ubiquitous across cultured cell lines and demonstrate dynamic motile and sensing properties. The expression of the morphogen Sonic Hedgehog (Shh) can affect the occurrence of cytonemes in ligand-producing cells. We show that Shh travels along cytonemes in vesicles with the Shh deployment protein Dispatched. Live cell imaging identified that cytoneme-based Shh deposits can activate the pathway in receiving cells within seconds of release. The use of MEM-fix along with live cell imaging assays, is now providing insight into the functionality and the extent cells use cytonemes for morphogen transport.

P1863/B102

Model for Dendritic Actin Network Formation, Distributed Turnover, and Structural Remodeling.

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The dendritic network of actin filaments provides the force for lamellipodial protrusions, driven by actin filament polymerization and branch generation by the Arp2/3 complex. Electron microscopy of keratocyte lamellipodia has revealed a network structure that varies with distance to the leading edge: a dense brushwork composed of short filaments near the leading edge is followed by longer and more linear filaments near the center and rear. Single molecule imaging experiments have shown that actin assembles throughout the lamellipodium and that actin disassembles within a few seconds after incorporation into the filament network, suggestive of frequent severing near the barbed ends. Prior modeling of FRAP and single molecule imaging experiments suggested that diffusing actin oligomers anneal to the network throughout the lamellipodium. To further investigate the precise mechanisms behind network remodeling and the role of the oligomer pool, we created a three-dimensional stochastic model at the filament level that includes mechanisms for polymerization, depolymerization, branching, capping, uncapping, severing, oligomer diffusion, annealing, and debranching. The model reproduces the +/- 35^{o} orientation pattern when branching occurs within 10^{o} of the lamellipodial plane as well as the density of branches, ends and length distribution near the leading edge for both fibroblast and keratocyte lamellipodia (that differ in the magnitude of retrograde flow and polymerization rates). We find that incorporating frequent severing near the barbed ends (occurring at a lower rate near the leading edge) and annealing, provides a mechanism for structural remodeling at the level observed in electron micrographs. This mechanism also leads to short actin lifetimes and distributed turnover, which we compare quantitatively to prior experiments.

P1864/B103

Discrete Mechanical Model of Lamellipodial Actin Networks.

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Determining how mechanical forces and actin filament turnover coordinate within the lamellipodium is important for understanding cell migration. Several computational and mathematical models have been used to investigate the overall stress profile of lamellipodial actin networks, including around focal adhesions, often using a continuum approximation. However, the forces and deformations of individual actin filaments important in lamellipodial mechanics have largely not been considered. We develop a

filament-level computational model of an actin network undergoing retrograde flow simulated via 3d Brownian dynamics. Retrograde flow is maintained in our simulations due to both pushing forces from the leading edge (due to actin polymerization) and pulling forces (due to molecular motors) on the actin network. We develop computational methods allowing us to simulate networks with densities similar to that in prior electron micrographs. Connectivity between individual actin segments is maintained by both permanent and temporary bonds meant to represent the Arp 2/3 complex and actin crosslinkers respectively. Remodeling of the network occurs via the addition of single actin filaments near the leading edge and via filament and bond severing of bonds under high stress. With our model we are able to investigate how several parameters affect the stress distribution, network deformation and retrograde flow speed of the actin network. We focus on varying the network connectivity, the strength of the pushing and pulling forces and the presence and location of nascent focal adhesions. Our model is able to quantitatively capture the experimentally observed reduction in retrograde flow speed when the network is exposed to cytochalasin D, which halts actin polymerization. We also find that increased levels of network connectivity lead to higher levels of stored stress within the network which leads to longer ranged network distortions when focal adhesions are encountered.

P1865/B104

Synaptic Control of Lysosome Trafficking in Dendrites.

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Organelle positioning within neurites is required for proper neuronal function. In dendrites with their complex cytoskeletal organization, transport of organelles is guided by local specializations of the microtubule and actin cytoskeleton, and by coordinated activity of different motor proteins. Here, we focus on the actin cytoskeleton in the dendritic shaft and describe dense structures consisting of longitudinal and branched actin filaments. As evident from the super-resolution imgaing data, these actin patches are devoid of microtubules and are frequently located at the base of spines, or form an actin mesh around excitatory shaft synapses. Using lysosomes as an example, we demonstrate that the presence of actin patches has a strong impact on dendritic organelle transport, as lysosomes frequently shortly pause or stall at these locations. We provide mechanistic insights on this pausing behavior, demonstrating that actin patches form a physical barrier for kinesin-2 driven cargo. In addition, we identify myosin Va as an active tether which mediates long-term stalling. Does synaptic activity control the switch between kinesin-2 and myosin Va driven transport? We are currently exploring the role calcium binding protein calmodulin and caldendrin in the regulation of myosin Va activity by employing various in vitro reconstitution and cellular assays. Taken together, we propose that the correlation between the presence of actin meshes and halting of organelles could be a general principle by which synapses control organelle trafficking.

P1866/B105

Rapid Treadmilling and Myosin Motors Synergistically Induce Formation of Cortex-like and Ring-like Actomyosin Structures.

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Semiflexible actin filaments crosslinked by myosin motors control cell motility and morphology via a dynamic remodeling process - treadmilling. Filaments in living cells are often found to be abundant at

the cell periphery, frequently forming ring-like structures or thin, contractile sub-plasma-membrane cortices. However, in less chemically dynamic reconstituted actomyosin networks, motor proteins induce global geometric contraction, creating cluster-like structures. The fundamental mechanisms underlying the emergence of cortex-like or ring-like actin networks in cells are not well understood. Here we used an advanced computational modeling platform, MEDYAN, which couples stochastic reaction-diffusion treatment with polymer physics and mechanics, to explore the interplay between filament treadmilling and the activity of non-muscle myosin II motors in confined systems. Our results indicate that the actomyosin network geometry is tightly regulated by filament treadmilling. In our simulations, slowly treadmilling actomyosin networks undergo geometric collapse and form clusters. On the other hand, enhancement of filament treadmilling generates cortex-like structures in 3D spherical networks and ring-like structures in quasi-2D disk-like networks, without the necessity to tether filaments to the boundary or preferentially nucleate filaments. We validated the effect of filament treadmilling on the ring-like structures of spreading T cells by using Latrunculin a (LatA) to inhibit actin filament assembly and monitoring filament dynamics via Total Internal Reflection Fluorescence microscopy. With high doses of LatA, actin rings in T cells experience centripetal collapse accompanied by the formation of clusters or bundles, which is consistent with our simulations mimicking the effects of LatA. We hypothesize that rapid treadmilling allows filaments to escape from global clustering and finally reach the network boundary, where myosin motors cause the collapse of these filaments into cortices and rings.

P1867/B106

Leading Edge Maintenance in Migrating Neutrophil-like HL-60 Cells Is an Emergent Property of Branched Actin Growth.

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Actin-driven, directional cell motility is one of the most fundamental behaviors in animal cell biology. To maintain polarized migration, cells must coordinate the stochastic growth of tens of thousands of nanometer-sized actin filaments over micron-scale distances along their leading edge. We employed high speed, high resolution microscopy of migrating neutrophil-like HL-60 cells to directly observe leading edge maintenance evolving over time. To our surprise, we discovered that cells' leading edge shape continuously undergoes dynamic sub-micron, sub-second undulations, all while maintaining nearly constant overall cell shape. Under the assumption that these shape fluctuations reflect the underlying actin dynamics driving migration, we set out to quantitatively characterize and then perturb shape dynamics in order to develop a deeper understanding of the mechanisms cells use to maintain leading edge shape, and thus polarized migration. Given that the overall leading edge shape is quite stable, we reasoned that any perturbation from the average shape would have a characteristic amplitude and relaxation timescale, and that this relaxation might have some wavelength dependence. Adapting well-established methods from polymer physics, we used Fourier mode time-autocorrelation analysis to investigate the relaxation of shape fluctuations over time. Surprisingly, we found that shape fluctuations at all wavelengths undergo oscillations as they decay, implying some memory exists in the system that causes fluctuations to "overshoot" as they relax, much like a Hookean spring. Furthermore, live-cell fluorescence imaging of HL-60 cells endogenously expressing GFP-β-actin revealed that actin density fluctuations also undergo oscillatory decay that is perfectly anticorrelated with leading edge

shape fluctuations. The rich behavior and quantitative nature of this dataset made it primed for physical modeling. We thus employed a swath of simple "toy" models to explore potential molecular mechanisms that could explain leading edge oscillations and their relationship to actin density. Remarkably, we found that a simple evolutionary filament orientation model (Maly and Borisy 2001) is sufficient to explain the oscillations. In this model, the network oscillates between a fast-growing, low density state in which filaments grow perpendicular to the leading edge, and a slow-growing, high density state in which filaments grow towards the membrane at approximately one half of the branching angle. Excitingly, these results suggest that branched actin growth against a membrane is inherently self-correcting and thus sufficient for maintaining leading edge shape without requiring higher-level regulation.

P1868/B107

Macromolecular Crowding Modulates the Organization of Actin Bundles Induced by Actin Crosslinking Proteins.

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Actin crosslinking proteins such as fascin and α-actinin form higher-ordered actin bundles that play critical roles in various cellular structures including filopodia and lamellipodia. Macromolecular crowding agents are widely used to understand the functional properties of proteins in intracellular environment. While the roles of actin crosslinking proteins in bundling have been well studied in dilute buffer conditions, how they affect bundle organization in crowded environments is not well established. We hypothesize that crosslinked actin bundles by fascin and α-actinin may be modulated under macromolecular crowded environment due to changes in interaction between crosslinking proteins and filaments. Here, we investigate how macromolecular crowding affects the organization of fascin- or α-actinin-induced bundles in *vitro and* in *silico.* Total internal reflection fluorescence (TIRF) microscopy and atomic force microscopy (AFM) imaging revealed that macromolecular crowding agents densely packed fascin-induced bundle and promoted different morphology of α-actinin-induced bundle depending on the type of crowders. Using co-sedimentation assay, we found that binding activity of fascin and α-actinin was affected in the presence of crowders. All-atom molecular dynamics simulation results supported that crowding agents modulate the binding interactions between actin and fascin or the CH1 domain of α-actinin. This work suggests macromolecular crowding affects the organization of actin crosslinking protein-induced bundles by modulating the interaction between actin crosslinking proteins and actin filaments.

P1869/B108

Spatiotemporal Dynamics of Actin and Microtubules in Drosophila Egg Chambers.

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Within a single cell, simple actin monomers assemble into structures with a remarkable range of geometries, dynamic properties and subcellular distributions, yet how this is achieved is poorly understood. We are using assembly of actin cables during oogenesis as a model to dissect these mechanisms. One of the last events in oogenesis is cytoplasmic dumping where a syncytial network of nurse cells contracts to expel their contents into the developing oocyte. At stage 10B just prior to

dumping, an array of actin cables initiates at the cell cortex and elongates toward the nucleus in each nurse cell. The cables continue to elongate after nuclear contact, pushing the nuclei away from the ring canals to prevent obstruction during dumping. We assessed the spatial distribution and dynamics of the growing cables and found that during stage 10B actin cables at nurse cell-nurse cell (N-N) and nurse cell-oocyte (N-O) junctions elongate at a rate of 0.11µm/minute. After nuclear contact, cable growth continues at a similar rate. While the majority of cables initiate within the first 45 minutes, new actin cables emerge through the first two hours of stage 10B. We further identified a population of actin cables at nurse cell-follicle cell (N-F) borders that can only be observed live. N-F cables grow significantly slower (0.06µm/minute) and contact the nucleus later than N-N or N-O cables, suggesting that different regulatory factors control their growth. Work from our lab and others has shown that Enabled (Ena), the formin Diaphanous (Dia), and its interactors Adenomatous polyposis coli-1 (APC1) and APC2 are required for proper assembly of actin cable arrays. Interestingly, while APC2 and Dia localize to the cortex at N-N, N-O, and N-F borders, Ena only associates with N-N and N-O borders. This suggests that Ena may be elongating the faster-growing N-N and N-O cables, and that N-F cables are Ena-independent. Consistent with that model, Ena reduction resulted in a reduced growth rate for N-N cables. We are currently assessing cable initiation and dynamics in other actin assembly mutants. In addition to a role for actin assembly factors, we found that acetylated microtubules (MTs) co-align with actin cables during their elongation, and that nocodozole inhibition of MTs blocks cable elongation. Stabilizing MTs with taxol causes actin cables to detach from the cortex. These results suggest that a dynamic MT network is required for actin cable elongation. We are currently investigating the role of MT +-TIP proteins in actin cable assembly (see poster by A. Leslie et al.). Taken together, our results suggest that many factors are orchestrated to control the spatiotemporal and dynamic properties of actin cable initiation and elongation.

P1870/B109

Kinesin-driven Microtubule Networks Control Dynamical Structure of Interpenetrating Actin Networks.

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Many of the fundamental physiological operations of the cell are accompanied by the fluid flow: locomotion, chromosome dynamics, cytoplasmic organization and, on an organismal level, embryogenesis. This dynamic reorganization is often generated by the microscale activity of molecular motors coupled to cytoskeletal proteins. The dense and diverse nature of the cytoplasm impedes our ability to interpret and predict this internally-driven motion. Although many of the major components in the cytoplasm are known, the dynamics of fluid flow and its coupling to physiological tasks is not. To capture the rich behavior of such living materials, we built a simple composite system of interpenetrating networks of bundled microtubules and entangled filamentous actin. The bundled microtubules are driven by the kinesin motors which generate flow. We show the activity of the microtubule bundles can fluidize and structurally remodel the interdigitated actin network. By controlling the mechanical properties of the actin network or the magnitude of fluid flow, we can transition the actin from a state of highly heterogeneous structures, characterized by large fluctuations in local density, to a state in which the actin is homogeneously distributed.

P1871/B110

Microtubules and the Microtubule +tips Eb1, Clip-170, and the Gas2 Protein Pigs Regulate Actin Cable Assembly and Organization during *Drosophila* Oogenesis.

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Crosstalk between the actin and microtubule (MT) networks plays essential roles in many cellular processes, but the mechanisms governing these interactions are not well understood. We are using actin cable assembly in *Drosophila* nurse cells as a model for dissecting actin-MT interactions. The nurse cells are a 15-cell syncytium connected to each other and to the developing oocyte through actin-based ring canals. Throughout oogenesis, the nurse cells transport mRNA, protein, and organelles into the oocyte via the ring canals. At stage 10B, actin cables emerge from the cortex and elongate toward the nucleus just prior to cytoplasmic dumping, a final rapid extrusion of nurse cell cytoplasm into the oocyte. The cables reposition the nuclei away from the ring canals to prevent obstruction. We discovered that acetylated MTs co-align with actin cables throughout their growth. Disrupting MTs with nocodozole blocks actin cable elongation, while stabilizing MTs with taxol results in cable detachment from the cortex and loss of further elongation. This suggests that a dynamic MT network is required for cable elongation. Further, disrupting actin cables through loss of the actin filament bundler Quail (Drosophila Villin) distorts MT organization, suggesting codependence of the networks. To begin to understand the molecular mechanisms of actin cable-MT crosstalk, we examined the role of the MT +TIP protein EB1, that we and others have shown can impact the actin cytoskeleton. Knockdown of EB1 resulted in premature cable assembly, and in early stages of assembly, significantly increased the number of cables, consistent with a role as an actin assembly inhibitor. In addition, loss of EB1 significantly reduced the actin cable growth rate and resulted in cables with significantly increased tortuosity, but did not impact actin-MT co-alignment. To dissect the complex role of EB1 in cable growth, we are interrogating two of its binding partners, CLIP170 and the actin-MT crosslinker Pigs (a Gas2 protein). We found that both CLIP170 and Pigs localize to puncta where actin cables and MTs co-align. In contrast to EB1 knockdown, loss of either CLIP170 or Pigs resulted in a significant decrease in actin cable number and length, suggesting a role for both proteins in promoting actin assembly. We are currently testing specific hypotheses to dissect the mechanisms by which EB1, CLIP170 and Pigs are modulating actin cable assembly.

P1872/B111

Determining Mechanisms of EPS8 Function during Microvilli Growth.

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Microvilli are actin-based protrusions evolutionarily conserved from the earliest animal cells to human intestinal epithelia. In the context of the human intestine, microvilli collectively constitute the intestinal brush border and serve as the sole site of nutrient absorption within the body. Despite persistence during evolution and the imperative role of microvilli for enabling human viability, little is known about the molecular events that underlie growth of the supporting microvilli actin bundles from the cell surface. Epidermal growth factor receptor pathway substrate 8 (EPS8) is an actin binding protein previously implicated in microvilli elongation and motility, and localizes specifically to the distal tips of microvilli in both mouse intestinal tissue and cell culture models. Moreover, previous studies have shown that loss of EPS8 leads to a decrease in microvilli length. As the barbed ends of actin filaments—the physiological sites of new monomer addition—are oriented towards the distal tips of microvilli, we propose that EPS8 functions in regulating actin bundle growth. Using the brush border inducible Ls174T-

W4 cell line, we demonstrate that EPS8 actin binding activity is necessary but insufficient for robust targeting to the distal tips of microvilli. Moreover, using spinning disk confocal microscopy, we are able to visualize individual microvilli growth events in kidney epithelial LLC-PK1-CL4 cells. These studies show that EPS8 puncta can predict sites of microvilli growth, and that microvilli length rapidly decreases shortly after EPS8 is lost from the distal tips. Intriguingly, we find that not only can microvilli growth occur *de novo* from the cell surface, but that new microvilli bundles can form orthogonally or at the base of existing microvilli, with EPS8 marking the new sites of actin bundle growth. Collectively, these results demonstrate that EPS8 is one of the earliest molecules localized to sites of microvilli growth and functions to stabilize growing actin bundles.

P1873/B112

Reconstitution of the Lamellipodia to Filopodia Transition Using Purified Proteins.

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Cells simultaneously assemble and maintain different actin filament (F-actin) networks that are involved in diverse processes such as cell motility and cell division. For example, filopodia are finger-like membrane protrusions composed of long F-actin bundles that have been proposed to sense extracellular chemical and mechanical signals to steer the cell. Two actin elongation factors, formin and VASP, localize to filopodia tips, driving their elongation. It has been hypothesized that filopodia can be generated from the lamellipodia, which consists of densely packed, branched actin filaments that are kept short by capping protein. How can filopodia emerge from this high density of capped filaments? Do different assembly factors facilitate the formation of diverse filopodia? to answer these questions, we reconstituted the actin cytoskeleton present at the leading edge of the cell in *vitro* using purified proteins. By combining micropatterning and bead motility assays with three-color Total Internal Reflection Fluorescence (TIRF) microscopy, we reconstituted the formation of filopodia-like networks (FLN) from densely-branched networks in the presence of actin, Arp2/3 complex, its activator pWA, and the bundler fascin. The addition of a saturating concentration of capping protein inhibits FLN formation, which can be rescued by the inclusion of the processive F-actin barbed-end elongator formin mDia2, and to lesser extent by VASP. Interestingly, formin-generated networks are relatively long and lacking capping protein, unlike the short VASP-generated networks. In addition, we discovered that the F-actin bundler fascin facilitates filopodia-like network formation by inhibiting Arp2/3 complex-mediated branching by 50 percent. Our biomimetic reconstituted system reveals that the combination of the F-actin bundler fascin with the actin elongation factors formin or VASP is sufficient to trigger a transition from a dense branched network of capped F-actin to a FLN. Importantly, the formation of these specific F-actin networks is established solely by the binding kinetics of these actin-binding proteins and does not require complex signaling pathways.

P1874/B113

Reconstitution of Dynamic Actin Cables from Purified Components.

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A central unanswered question in biology is how cells assemble large protein structures that have precise sizes and shapes tailored to their functions and simultaneously undergo dynamic turnover. *Saccharomyces cerevisiae* actin cables provide an ideal system for addressing this question, because

their essential function in cargo transport demands that they simultaneously undergo rapid assembly and turnover while maintaining well-defined lengths. Genetic studies have identified a molecular parts list required for proper cable morphology and function in *vivo*, but a minimal set of components sufficient to assemble a dynamic cable has not yet been established, and it has remained unclear how each component contributes to cable formation. Here, using only eight purified *S. cerevisiae* proteins (actin, profilin, formin, capping protein, tropomyosin, cofilin, coronin, and AIP1), we were able to assemble linear actin structures that undergo steady-state polarized turnover while maintaining lengths similar to cables in *vivo*. Cables were polymerized at one end by formins attached to beads, in a profilin-dependent manner, and disassembled at the other end by cofilin, coronin, and AIP1. Cable assembly also required tropomyosin, which antagonized disassembly-promoting factors. Capping protein restricted actin polymerization to the formin-coated beads and limited cable network density. Remarkably, removal of individual proteins from the in *vitro* system led to cable defects similar to the phenotypes observed in *vivo* for disrupting the corresponding genes. Thus, our reconstitution system closely mirrors cellular requirements for cable formation and demonstrates that a minimal set of nanoscale components can self-organize into a dynamic, microns-long cellular structure.

P1875/B114

Mechanically Regulated Structural States of F-actin.

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The ability of cells to sense mechanical forces in their microenvironments influences cellular functions including migration, proliferation, and apoptosis. The actin cytoskeleton, a network consisting of actin filaments (F-actin), myosin motor proteins, and over 150 Actin Binding Proteins (ABPs), plays a central role in cellular mechanics. F-actin has been reported to transduce mechanical stimuli into downstream biochemical pathways (“mechanotransduction”) through force-regulated interactions with mechanosensitive ABPs. As multiple actin subunit conformations have been reported to co-exist in filament populations in the absence of force**,** we hypothesized that actin’s inherent structural plasticity facilitates the formation of force-regulated structural states which can be recognized by mechanosensitive ABPs. To test this hypothesis, we developed a novel reconstitution system utilizing surface-immobilized myosin motor proteins to study the conformational response of F-actin to compressive and tensile forces with cryo-electron microscopy (cryo-EM). Utilizing this system for cryo-electron tomography (cryo-ET) studies, we observe that F-actin adopts a super helical spiral conformation in local regions along filaments (which we term “squiggles”) in the presence of both tension and compression. An alysis of the distribution of subunit positions in tomograms suggest that squiggles form due to asymmetric longitudinal promoter spacing between the two strands that compose the filament. We furthermore observe the frequent presence of dislocated subunits in squiggles, which we posit are the initiating sites of lattice defects that propagate along a strand. In addition, to providing the first direct visualization of a force-induced rearrangement of F-actin, our studies identify a potential binding platform for mechanosensitive ABPs.

P1876/B115

Intrinsic Constraint of the Phenotypic Plasticity of the Actin Cytoskeleton Reveals Limited Attractor States.

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Numerous proteins and pathways regulate F-actin organisation. In combinatorial terms, this suggests that an almost unlimited number of regulatory states are conceivable. Consequently, the potential for plasticity in F-actin phenotypes appears virtually unbounded. To estimate the actual degree of F-actin phenotype plasticity quantified by 74 parameters, we used a library of 114,400 structurally diverse compounds to induce unbiased chemical perturbations. Remarkably, we estimated the number of recurrent F-actin phenotypes that emerged to be approximately 25. This predicts that compounds with distinct molecular mechanisms induced equivalent phenotypes, suggesting that these recurring phenotypes reflect a low number of equilibrium or attractor states in actin organisation. Compounds which produce a Latrunculin A-like F-actin phenotype use at least three different pathways. Similarly both a talin activation phenotype and an anti-tropomyosin Tpm3.1 phenotype are produced by compounds using multiple pathways. This was further supported by dynamic analyses comparing phenotype trajectories over time, showing how initially divergent phenotypes ultimately converged into equivalent end-states. We propose that infrequent attractor states in the actin phenotypic landscape reflect a channelling of high perturbative diversity into low phenotypic variety. The existence of such attractor states may have provided a mechanism to suppress chaotic outcomes during the evolution of this complex, functionally integral system.

P1877/B116

Bending of Actin Filaments into Rings by Iqgap Family of Proteins.

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The acto-myosin cortex in cells plays a central role in providing mechanical stability, enabling vesicle transport, cell migration and cell division. This versality is achieved by interaction with many regulatory and actin binding proteins and the interplay with the plasma membrane to locally control actin filament length, stability and crosslinking to other filaments. Form and function go together as, e.g., filopodia are formed by parallel bundles of actin filaments generated by α-actinin and membrane anchored formins. Here, we report about a new module (Curly) within IQGAP family proteins that binds actin and leads to the bending of individual actin filaments into tight rings when anchored to a lipid membrane in vitro. We identified the Curly module within IQGAP1 (*H. sapiens*), Rng2 (*S. pombe*) and Iqg1 (*S. cerevisiae*). Membrane anchored Curly induces reproducible and efficient formation of F-actin rings with a characteristic curvature (0.5-1 μm^{-1}) and recognizes F-actin orientation. Interestingly, decoration of F-actin with the tropomyosin increases actin ring formation, while Fimbrin abrogates it. Addition of myosin II filaments results in further formation of actin rings by induced f-actin flow and, more importantly, in robust ring contraction. This constitutes, hence, a minimal (four components) contractile ring system. The discovery of Curly provides a new opportunity to address and control F-actin curvature in cells and in vitro and will open new approaches to generate synthetic cells capable of division and controlled shape changes.

Dynein

P1878/B118

A Novel Function of Nuclear Transport Factor Kpna1/importin Alpha5 in Neuronal Cells.

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Schizophrenia, autism spectrum disorder (ASD), and attention deficit/hyperactivity disorder (ADHD) have been reported to be caused by intracellular transport deficits in neuronal cells and their migration disorder. However, the intracellular transport systems have not been studied in this context. Since importins mediate transportation from cytoplasm to nucleus, and from synapse to soma, perturbation of importin-dependent pathway may have significant neuronal consequences. Our behavioral tests using KPNA1 knockout (KO) mice revealed impairment of novel object recognition, tendency of depression, and increased sensitivity to phencyclidine (PCP), a non-competitive antagonist for NMDA glutamate receptor that causes schizophrenia-like symptoms. Coupling intracellular signals to behavioral output likely requires clarification of a novel functional role of KPNA1 in neuronal cells. In this study, DNA microarray data provided insights into the possible gene expression alterations in parts of brain, such as prefrontal cortex (PFC), and nucleus accumbens (NA) of KPNA-KO mice. Principal components analysis (PCA) of KPNA-KO mice with PCP revealed different clusters in scatter plots, suggesting higher sensitivity and/or weakness to PCP. Notably, gene expression of cytoplasmic dynein components and their associated factors was found to be reduced in KPNA-KO mice, which was further, remarkably reduced in presence of PCP. Live-cell imaging using FRAP (fluorescence recovery after photobleaching) and dual fluorescence tracking showed migration of KPNA1 both antero- and retrogradely, along with co-migration of cytoplasmic dynein. Overexpressed KPNA1 accumulated near the centrosome and around the nucleus. Our findings suggested that KPNA1 functions in intracellular transport through microtubules (MTs) and in neuronal cell migration dependent on MT traction. This unexpected and intriguing discovery, related to axonal transport, may provide new insight into neuropsychiatric disorders.

P1879/B119

Role of Coiled-coil Registry Shifts in Activation of Human Bicaudal D2 for Dynein Recruitment Upon Cargo-binding.

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Dynein adaptors such as Bicaudal D2 (BicD2) recognize cargo for dynein-dependent transport and cargo-bound adaptors are required to activate dynein for processive transport, but the mechanism of action is unknown. Here, we report the X-ray structure of the cargo-binding domain of human BicD2 and investigate structural dynamics of the coiled-coil (1). Our molecular dynamics simulations support that BicD2 can switch from a homotypic coiled-coil registry, in which both helices of the homo-dimer are aligned, to an asymmetric registry, where a portion of one helix is vertically shifted, as both states are similarly stable and defined by distinct conformations of F743. The F743I variant increases dynein recruitment in the Drosophila homolog whereas the human R747C variant causes spinal muscular atrophy. We report spontaneous registry shifts for both variants, which may be the cause for BicD2-hyperactivation and disease. We propose that a registry shift upon cargo-binding may activate auto-inhibited BicD2 for dynein recruitment (1). Furthermore, we report the x-ray structure of the C-terminal

domain of the *Drosophila* Bicaudal variant of BicD which provides experimental support for a coiled-coil registry shift, which may be the underlying cause of the observed hyperactivation of BicD in the variant (unpublished data). 1. Noell CR, Loh JY, Debler EW, Loftus KM, Cui H, Russ BB, Zhang K, Goyal P, **Solmaz** SR. J Phys Chem Lett, 2019 in press. doi: 10.1021/acs.jpclett.9b01865.

P1880/B120

Coiled-coil Regsitry Shifts in the F684I Mutant of Bicaudal Result in Cargo-independent Dynein Recruitment.

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Dynein adaptors such as *Drosophila* Bicaudal (*Dm* BicD) recognize cargo and are required to activate dynein for processive transport. In the absence of cargo, auto-inhibited BicD cannot recruit dynein, but the underlying mechanism of cargo activation is elusive. We show with single molecule processivity assays that auto-inhibition is abolished in the full-length Bicaudal mutant F684I, which activates dynein motility in the absence of cargo. To investigate the structural basis for activation, we determined the X-ray structure of the C-terminal cargo-binding domain (CTD) of *Dm* BicD-CTD/F684I. The mutant has a homotypic coiled-coil registry, in which the two helices of the homo-dimer are aligned at equal height, however, an N-terminal ~20 residue region is disordered for one of the two chains, and therefore the coiled-coil registry in that region cannot be assigned. Thus, the F684I mutation results in the formation of multiple conformations and induces flexibility. In contrast, the wild type structure has an asymmetric coiled-coil registry, in which the two chains are vertically shifted by ~ one helical turn in a portion of the coiled-coil. We propose that a coiled-coil registry shift upon cargo binding activates BicD for dynein recruitment. Interestingly, the human homolog of the Bicaudal mutant, BicD2-CTD/F743I, shows diminished binding of the cargo Nup358 compared with the wild type, while binding of the cargo $Rab6^{GTP}$ is unaffected. Therefore, we propose that a coiled-coil registry shift also modulates cargo selection for BicD2-dependent transport pathways in *mammaliae*, which are important for brain development, faithful chromosome segregation and signaling.

P1881/B121

Characterization of Human Disease Mutations of Dynein Adaptor Bicaudal D2 Provides Insights into Possible Causes for Spinal Muscular Atrophy.

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Dynein adaptor proteins such as Bicaudal D2 (BicD2) are an integral part of the dynein transport machinery as they select cargoes for transport along the minus ends of microtubules and link them to dynein motors. In the absence of cargoes BicD2 forms an auto-inhibited state that cannot recruit dynein. The importance of these transport pathways for brain and muscle development is reflected in the fact that human disease mutations cause devastating brain and muscle development diseases, including spinal muscular atrophy. Here, we use an approach that combines structural methods, molecular dynamics (MD) simulations and circular dichroism (CD) spectroscopy to establish how these disease mutations modulate the coiled coil structure of BicD2. We also present new data supporting the idea that cargo-binding induces a coiled coil registry shift in human BicD2 which activates it for dynein recruitment. Our structural analysis indicates that the human disease mutations R747C and E774G

destabilize the coiled coil structure. CD wavelength spectra indicate that these disease mutations result in misfolding. In MD simulations, the R747C disease mutation induces a transient and local coiled-coil registry shift. We thus hypothesized, that combining the disease mutation with a second mutation (F743I), which induces spontaneous coiled-coil registry shifts in MD simulations, could rescue folding and stabilize BicD2 in a registry-shifted conformer. Of note, this mutation results in increased dynein recruitment in the *Drosophila* homolog. This R747C/F743I double mutant was characterized by CD wavelength scans, which indicate that the R747C/F743I double mutation rescues folding of BicD2. The double variant has a higher melting temperature than the BicD2 wild type and the double mutation also shows comparable binding to the cargo $Rab6^{GTP}$ as the wild-type.We conclude that the human disease mutations E774G and R747C destabilize the coiled coil structure of the cargo binding domain of BicD2 and may result in context of the full length protein either in complete or partial loss of function, which may be an underlying cause for spinal muscular atrophy in these mutants. We also conclude that BicD2-CTD R747C/F743I double mutant is thermodynamically more stable than the wild type, and binds cargoes similarly to the WT. This variant may be stabilized in a registry-shifted conformer, Thus, we provide evidence that disease-causing mutations in the BicD2 coiled-coil may alter the equilibrium between registry-shifted conformers, which we propose as a mechanism of pathogenesis that may also apply to other coiled-coils.

P1882/B122

Septin 9 (SEPT9) Promotes the Retrograde Transport of Endolysosomes by Scaffolding Dynein-Dynactin Complexes.

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Spatiotemporal coordination of membrane traffic is paramount for cell development and survival. Long-range transport of membrane cargos is driven by the microtubule motors kinesin and dynein. However, how cargos selectively associate with and activate motors is not well understood. Here we find that membrane-associated SEPT9, a member of the septin family of GTPases, regulates dynein-dependent transport of lysosomes by direct association with dynein and dynactin. We examined the abundance of septins on cellular membranes by density gradient fractionation and we found that SEPT9 is enriched in lysosomal fractions. Super-resolution structured illumination microcopy (SIM) showed that SEPT9 localizes on domains of Lamp1-positive endolysosomes. In the axons of cultured hippocampal neurons SEPT9 co-migrates with Lamp1-positive endolysosomes, which move with a retrograde bias toward the cell body, and SEPT9 overexpression in COS-7 fibroblasts results in perinuclear repositioning of lysosomes. Membrane relocation assays showed that ectopic targeting of SEPT9 to mitochondrial membranes is sufficient to induce perinuclear clustering of mitochondria, which is reversed by nocodazole treatment and p50/dynamitin overexpression. Similarly, coupling of SEPT9 to peroxisomes through a rapalog-induced heterodimerization assay resulted in perinuclear clustering of peroxisomes. Biochemical in *vitro* binding assays showed that recombinant SEPT9 interacts directly with native dynein/dynactin isolated from HEK-293 cells. We mapped dynein interaction to the conserved GTPase domain of SEPT9, while the N-terminal domain of SEPT9 interacts with dynactin. Interestingly, recombinant SEPT9 interacts with the N-terminal adaptor-binding region of dynein intermediate chain (DIC) (108-268aa in mouse IC1A), which does not associate with SEPT2/6/7. Strikingly, this interaction is sensitive to the nucleotide state of SEPT9 as SEPT9 association with DIC is diminished in the presence of the non-hydrolyzable GTP analog GTPγS. Collectively, our findings suggest that SEPT9 functions on the membranes of endolysosomes as a scaffold for the recruitment and potential activation of dynein. On-

going work is exploring this hypothesis and testing the role of SEPT9 in lysosome positioning under conditions of cell stress.

P1883/B123

Dynactin P150 Promotes Processive Motility of DDB by Minimizing Diffusional Behavior of Dynein.

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Dynactin p150 promotes processive motility of DDB by minimizing diffusional behavior of dynein

Cytoplasmic dynein is the principal minus-end directed transport motor in cells. In *vitro*, cytoplasmic dynein motors are shown to be activated by adaptor proteins like BicD2, by forming a dynein-dynactin-BicD2 complex, DDB. A complete understanding of dynein function and regulation requires characterizing the role of the different components of the DDB complex. The dynactin subunit p150, which contains a flexible charged domain, interacts with microtubules and is proposed to act as diffusive tether that enhances processivity of dynein-dynactin. However, how dynein is activated in DDB complexes and the contribution of p150 to dynein motility in the context of the DDB complex remain unclear. We used single-molecule microscopy to analyze movement of DDB complexes along immobilized microtubules. In contrast to kinesin, DDB complexes display diverse motility behaviors that include pauses, diffusive episodes and processive runs having a range of minus-end velocities. To characterize switching between processive, diffusive and stuck states, we used Interferometric Scattering (iSCAT) microscopy to track DDB at 100 frames/s and developed a regression-based algorithm to classify switching between states. DDB spends 65 % of its time undergoing processive stepping and 4% of its time undergoing 1D diffusion along the microtubule. The role of p150 was tested by characterizing the motility in the presence of a function-blocking p150 antibody. Contradictory to predictions based on p150 acting as a diffusive tether, we found instead that blocking p150 enhanced the fraction of time DDB spends diffusing on the microtubule to 17%, and reduced the processive fractional duration to 55%. Thus, the diffusive behavior of DDB is likely due to dynein switching into an inactive (diffusive) state rather than p150 tethering DDB to the microtubule. The data suggest a model of DDB in which, rather than simply acting as a diffusive tether, interaction of dynactin p150 with the microtubule promotes or stabilizes an active conformation of dynein and therefore promotes processive minus-end motility during bidirectional transport in cells.

P1884/B124

Pac1/LIS1 Promotes an Uninhibited Conformation of Dynein That Coordinates Its Localization and Activity.

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Cytoplasmic dynein is a minus end-directed microtubule motor that transports myriad cargos in various cell types and contexts. How dynein is regulated to perform all these activities with a high degree of spatial and temporal precision is unclear. Recent studies have revealed that human dynein-1 and dynein-2 can be regulated by a mechanism of autoinhibition, whereby intermolecular contacts limit motor activity. Whether this autoinhibitory mechanism is conserved throughout evolution, whether it can be affected by extrinsic factors, and its precise role in regulating cellular dynein activity remain unknown. Here, we use a combination of negative stain EM, single molecule motility assays, genetic, and cell biological techniques to show that the autoinhibitory conformation is conserved in budding yeast, and it plays an important role in coordinating dynein localization and function in cells. Moreover,

we find that the Lissencephaly-related protein, LIS1 (Pac1 in yeast) plays an important role in regulating this autoinhibitory conformation of dynein. Specifically, our studies demonstrate that rather than inhibiting dynein motility, Pac1/LIS1 promotes dynein activity by stabilizing the uninhibited conformation, which ensures appropriate localization and activity of dynein in cells.

P1885/B125

Lis1 Promotes the Formation of Activated Cytoplasmic Dynein-1 Complexes.

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Cytoplasmic dynein-1 is a molecular motor that drives nearly all minus-end-directed microtubule-based transport in human cells, performing functions ranging from retrograde axonal transport to mitotic spindle assembly. Mammalian dynein is not a processive motor on its own. Activated dynein complexes consist of one or two dynein dimers, the dynactin complex, and an “activating adaptor”, with faster velocity seen with two dimers present. Mammalian dynein in the absence of these other components adopts a conformation known as “Phi”. Phi dynein is autoinhibited and cannot interact with microtubules productively. The current model for dynein activation proposes that Phi dynein must first adopt an “Open” conformation and then ultimately a “Parallel” conformation that is observed when it is bound to dynactin and an activating adaptor. Little is known about how dynein switches between the autoinhibited Phi conformation and the Open and Parallel conformations that lead to the assembly of the activated dynein complex. The highly conserved dynein binding protein Lis1 is required for nearly all of dynein’s known functions. Lis1 increases the binding of mammalian dynein to microtubules and increases the velocity of activated dynein complexes containing the activating adaptor BicD2. How Lis1 exerts these effects is unknown. Here, using in vitro reconstitution of recombinant human proteins and single-molecule imaging, we uncovered a novel role for Lis1 in the formation of activated dynein/dynactin complexes containing two dynein dimers. Lis1 is required for increased velocity of complexes activated by proteins representing three different families of activating adaptors: BicD2, Hook3, and Ninl. Once activated dynein complexes have formed, they do not require the presence of Lis1 for sustained increased velocity. In addition, using cryo-electron microscopy we show that human Lis1 binds to dynein at two sites on dynein’s motor domain, similar to yeast dynein. We propose that the ability of Lis1 to bind at these sites may function in multiple stages of assembling the motile dynein/ dynactin/ activating adaptor complex. We also propose a unifying model for how Lis1 function is conserved from yeast to humans.

P1886/B126

Impact of a Disease-Associated DCTN4 (p62) Variant on the Dynactin/Dynein Interactome.

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A single nucleotide variant in the dynactin component DCTN4 (rs35772018; hereafter DCTN4-018) is associated with enhanced pathology in cystic fibrosis and acute lung injury. We have shown that cells expressing DCTN4-018 exhibit defective cell migration, altered cortical actin dynamics and impaired integrin recycling, phenomena that are important for lung repair and airway health under a wide range of conditions. DCTN4 lies within the dynactin domain that allows dynein to bind to its various cargoes via coiled-coil adaptor proteins. Adaptors make direct contact with DCTN4, so we propose that the DCTN4-

018 variant (Y270C) alters dynactin binding. To identify adaptors and other dynactin-associated proteins that contribute to the DCTN4-018 phenotype we obtained the differential interactome between DCTN4-WT and DCTN4-018 using proximity-dependent biotin ligation (BioID). 018-DCTN4-BioID-3xFLAG was generated from a parent WT DCTN4 construct [provided by S.L. Reck-Peterson, along with LIC1-BioID line], and we made three HEK293 Flp-In cell lines: BioID control, WT-DCTN4-BioID, and 018-DCTN4-BioID. Similar amounts of WT and 018 DCTN4 incorporate into the dynactin complex as judged by pulldowns and biotinylation of other dynactin components, but sucrose gradient sedimentation and quantitation of DCTN4 auto-biotinylation reveal more unincorporated 018-DCTN4-BioID vs. WT. The different sizes of the free DCTN4 pool in two cell lines may yield different biotinylation levels which would complicate dataset analysis, so we also generated a dynein light intermediate chain 1 (LIC1)-BioID cell line expressing DCTN4-018 using CRISPR mutagenesis. These WT and 018 DCTN4 lines contained the same ratio of incorporated vs. free LIC1-BioID and, as expected, DCTN4 biotinylation levels were identical in the two lines. To eliminate background from proteins that might bind streptavidin beads indirectly via interactions with authentic dynactin-binding proteins, we used the DiDBiT method to enrich for biotinylated peptides. Peptides were TMT-tagged for multiplexing and relative quantitation by MS, identifying 765 and 789 proteins from the DCTN4-BioID and LIC1-BioID datasets, respectively. Dynein/dynactin components, as well as known adaptors were enriched over controls. A number of differential interactors relevant to the DCTN4-018 phenotype were also identified. Furthermore, our ability to precisely localize sites of biotinylation on dynactin components in the WT/018 DCTN4 BioID dataset allowed us to obtain novel information on dynactin structure. In summary, we have identified the first differential interactome of a disease-associated dynactin variant. Further evaluation will likely identify novel therapeutic targets for individuals expressing DCTN4-018.

P1887/B127

Dynactin Controls Movement of Different Endocytic and Post-golgi Carriers Via Distinct Protein-protein Interactions.

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Dynactin is a component of the essential microtubule-based motor, cytoplasmic dynein, that allows it to associate with different intracellular cargoes. High resolution cryo-EM has revealed the structural basis by which dynactin binds dynein via cargo-associated “activating adaptor” proteins. However, the molecular contacts that underlie dynactin-adaptor binding have not been defined, and how adaptor binding is regulated remains completely unknown. We showed previously that dynactin’s p25/p27 (DCTN5/DCTN6) components are required for normal motility of transferrin (Tfn)-labeled early and recycling endosomes but not conventional late endosomes. Here, we report that p25/p27 depletion suppresses formation of tubules that recycle proteins (CD98 and CD147) entering the cell via clathrin-independent endocytosis (CIE). p25/p27 depletion, or treatment with the dynein inhibitor dynarrestin, also interferes with segregation of CIE cargoes from Tfn-positive structures. By contrast, motility and tubule formation of post-Golgi cargoes (Lumenal-GFP or GFP-Rab6a’) is enhanced by these treatments, indicating that dynein-based motility can either permit or suppress movement of different endomembranes. We used mutagenesis to investigate how different p25 structural motifs contribute to the motile behavior of these cargoes. P25 contains a cluster of lysine residues in a surface loop (K74, K75, K78) that are predicted to interact electrostastically with adaptors, as well as a conserved C-terminal alpha-helix of unknown function. Using p25 knockdown followed by rescue with various

mutants, we found that Tfn motility and/or recycling was altered by changes in either the surface loop (specifically K78A) or C-terminal helix (deletion). CIE cargo behavior was altered by K78A only. Post-Golgi carrier movement is independent of K78 and the C-terminal helix but requires K74, which we also find is required for dynein-dynactin binding to the canonical activating adaptor BICD2. Taken together, these results suggest that p25 interacts with different cargo adaptors via a series of distinct contacts that mediate cargo selection and dynein-based movement.

P1888/B128

Distinct Populations of Cortical an choring Protein Num1 Mediate Mitochondria Tethering and Dynein-based Spindle Movement.

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Correct positioning of the mitotic spindle is essential for high-fidelity transmission of genetic material to daughter cells and is crucial for a wide range of processes including creation of cellular diversity during development and maintenance of adult tissue homeostasis. From budding yeast to human, spindle positioning involves attachment of the minus-end-directed motor cytoplasmic dynein to the plasma membrane, where it exerts pulling force on astral microtubules emanating from the spindle poles. Prior work has conceptualized that dynein attaches to cortical clusters containing the anchoring molecule NuMA (in human) or Num1 (in yeast), whose clustering activity is required for generation of dynein-based spindle-pulling forces. However, how clustering is regulated for proper dynein activity is unknown. Here we establish a role for Mdm36 in promoting the assembly of Num1 cortical clusters in budding yeast. Overexpression of Mdm36 dramatically enhances Num1 clustering, resulting in two morphologically distinct populations of Num1 patches that perform separate functions: one for mitochondria-tethering, whereas the other for dynein-dependent spindle-pulling function. Surprisingly, molecule counting assays revealed that the clusters mediating dynein-pulling function contain only ~ 6 copies of the Num1 protein, approximately 4 times lower than the levels in wild-type cells. Moreover, disrupting Num1 clustering (using *num1^{3E}* allele) affects mitochondria-tethering but not dynein-dependent spindle-pulling function. Additionally, we found that cluster enhancement does not correlate with an increase in cortical dynein activity, in contrast to the prevailing role of clustering for cortical dynein anchor. Our results indicate that Num1 clusters containing a small protein copy number is sufficient for dynein attachment and generation of spindle pulling forces.

P1889/B129

Linking Structure to Mechanochemistry for the Dynein-like AAA Protein Midasin.

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Midasin is an essential and highly conserved molecular machine that drives ribosome biogenesis by mechanochemically removing multiple assembly factors from the maturing preribosome. This massive (>500 kDa) AAA protein has an N-terminal region that bears resemblance to dynein, with six tandemly expressed AAA subunits, and a C-terminal region that bears resemblance to integrin, with a substrate-binding MIDAS domain. While midasin's general role in budding yeast ribosome biogenesis has been delineated, the molecular mechanism by which it converts chemical energy to mechanical force to reshape the preribosome remains unknown. Here, using a combination of structural and functional

assays with recombinant proteins, we show that despite being dynein-like in structure, midasin employs a unique mechanism for force production and transmission. Using cryo-electron microscopy in the presence and absence of the midasin-specific inhibitor Rbin-1, we show that the AAA ring of midasin is capable of undergoing large conformational changes that generate a transient site at which the distal MIDAS domain can directly bind. With truncated constructs, we show that this intramolecular binding interaction can be recapitulated in trans with nanomolar affinity. Using native mass spectrometry and single-molecule methods, we investigate how the MIDAS-ring interaction is gated by ATP and drug binding at the six available AAA sites. Overall, these data further our understanding of the midasin mechanochemical cycle.

P1890/B130

REg.

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Tau is a neuronal microtubule-associated protein which aggregates in Alzheimer's disease and other tauopathies. Tau is often described as a microtubule-stabilizing protein, but multiple lines of evidence suggest that this is not its primary function. Other roles of tau in neurons remain open to question. Studies in isolated squid axoplasm have found that aggregates of tau, or truncated tau monomers that expose the N-terminal, selectively inhibit anterograde fast axonal transport (FAT) through a mechanism dependent on amino acids 2-18. These amino acids comprise a phosphatase activating domain (PAD) which activates PP1 and GSK3β causing release of vesicles from kinesin motor protein. Whilst tau has a dynamic secondary structure it has been shown that unphosphorylated monomers adopt a "paperclip" conformation concealing the N-terminal. Post-translational modifications such as phosphorylation can stabilise more extended structures and expose domains at either the N- or C-terminals. Consequently, phosphorylation at the AT8 epitope (S199, S202, T205) in the central proline rich domain of tau also inhibits anterograde FAT through exposure of the PAD, and phosphorylation of S422 inhibits FAT in both directions by means of a currently unknown mechanism. We therefore hypothesize that tau has a physiological function in control of FAT, regulated by precise site-specific changes in its phosphorylation. Using video enhanced-differential interference microscopy in the squid axoplasm model, we show that individual pseudophosphorylation at each of the AT8 sites affects axonal transport differentially with S199E inhibiting both directions of FAT, S202E having no affect, and T205E inhibiting only anterograde FAT. In further experiments we use an antibody, TNT1, which binds specifically to the tau PAD, to test the dependence of these effects on exposure of the PAD. TNT1 successfully blocked inhibition of anterograde FAT by T205E tau but did not prevent inhibition of FAT by S199E tau, suggesting that T205E exposes the PAD but S199E does not. Combinations of phosphorylation at two sites (S199E+S202E, S202E+T205E, T212E+S214E) also inhibit FAT in both directions and could not be blocked by TNT1. These results strongly suggest that differential phosphorylation of tau can activate distinct downstream signaling pathways to affect both directions of fast axonal transport. Consistent with our hypothesis, we show that the PAD is exposed at specific sites linked to cargo delivery in developing hippocampal neurons in *vitro*.

P1891/B131

Using Computational Approaches to Reveal Mechanisms of Molecular Motors.

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Electrostatic interactions play important roles in many biology phenomena. Therefore, a lot of efforts have been made to model the electrostatic interactions in biological systems. However, it is extremely challenging to accurately calculate the electrostatic interactions in large biological systems such as dynein and viral capsid. Dynein, a molecular motor, is important for cargo transportation and force generation in cells. Dysfunction of dynein is associated with many diseases, such as ciliopathies, lissencephaly and other neurodegeneration disorders. A novel multi-scale simulation approach was used to study dynein's motion along microtubules. The results reveal that the electrostatic interactions play significant roles in dynein's motilities and functions along microtubule. Understanding the fundamental mechanisms in molecular motors sheds light on treatment of molecular motor related diseases.

P1892/B132

Coiled Coil Registry Shifts Modulate Cargo Selection of the Dynein Adaptor Protein BicD2.

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Dynein adaptors such as Bicaudal D2 (BicD2) recognize cargo for dynein-dependent transport. BicD2-dependent transport pathways are important for processes such as signaling, faithful chromosome segregation and brain development. The C-terminal domain (CTD) of human BicD2 recognizes two cargoes: the nuclear pore protein Nup358 (for transport of the nucleus), and $Rab6^{GTP}$(for transport of Golgi-derived vesicles) but the underlying mechanism of how the correct cargo is selected at the correct time is elusive. In the absence of cargo, BicD2 is auto-inhibited and unable to recruit dynein. Autoinhibition is compromised by a F743I mutation in the *Drosophila (Dm)* homolog. We have determined the X-ray structure of the *Dm* BicD-CTD/F684I mutant, which has a conformation with a homotypic coiled coil registry in which the two helices of the homo-dimer are aligned at equal height, whereas the wild type structure has an asymmetric coiled-coil registry in which the two chains are vertically shifted by ~ one helical turn in a portion of the coiled-coil. The F743I mutation also induces a spontaneous coiled coil registry shift in molecular dynamic simulations of Human BicD2-CTD. A registry shift is expected to remodel the surface of the coiled coil of BicD2 which harbors binding sites for cargoes, therefore, we investigated whether a coiled-coil registry shift plays a role in cargo selection of BicD2. Notably, Nup358 binds to a larger binding site compared to $Rab6^{GTP}$ that includes a region that is expected to undergo coiled-coil registry shifts. Interestingly, the human homolog of the Bicaudal mutant, BicD2-CTD/F743I, shows diminished binding of the cargo Nup358 compared with the wild type, while binding of the cargo $Rab6^{GTP}$ is unaffected. There are differences in the electrostatic surface potential of the F743I mutant compared to the wild-type structure in the area where Nup358 binds, which may be responsible for the observed differences in cargo binding. Therefore, we propose that a coiled coil registry shift modulates cargo selection for BicD2-dependent transport pathways in Mammalia.

P1893/B133

Dynein Containing an AAA3 ATP-hydrolyzing Mutation Is Activated by Dynactin Without Lis1 Or the Cargo Adapter HookA.

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Dynein cargo adapters and dynactin not only link dynein to cargo but also activate dynein (reviewed by Reck-Peterson et al., 2018 and by Olenick and Holzbaur 2019). This activation requires a switch of the dynein dimer from an autoinhibited "phi" conformation to an open and then parallel conformation allowing dynein to walk on microtubule (Zhang et al., 2017). Recent studies suggest that the dynein regulator LIS1 promotes the shift of dynein from the autoinhibited phi state to an uninhibited state (Elshenawy et al., 2019; Htet et al., 2019; Marzo et al., 2019; Qiu *et al.,* 2019). In *Aspergillus nidulans*, dynein and dynactin relocate from the microtubule plus ends to minus ends near septa upon activation by the early endosome adapter HookA. This requires LIS1, and this requirement is partially bypassed by the phi-opening dynein mutations. Since the function of LIS1 is also partially bypassed by the AAA4 arginine finger mutation implicated in AAA3 ATP hydrolysis, we performed an analysis on AAA3 ATP-binding (Walker a or wA) and ATP-hydrolyzing (Walker B or wB) mutants. We found that AAA3-wA dynein locates at the hyphal-tip together with abnormally accumulated early endosomes and also at septa without early endosomes. Loss of HookA or LIS1 causes the wA dynein to accumulate at the microtubule plus ends, while loss of the Arp1 subunit of the dynactin complex causes the wA dynein to become diffused in the cytoplasm. In contrast, the AAA3-wB dynein is strongly accumulated at septa without early endosomes, and this is independent of LIS1 or HookA. However, upon loss of Arp1 of dynactin, the wB dynein mainly decorates microtubules. These results suggest several interesting points: (1) Although previous in vitro studies showed that AAA3 ATP hydrolysis is crucial for dynein motility unless tension is applied to the linker domain of dynein (Bhabha et al., 2014; DeWitt et al., 2015; Nicholas et al., 2015), blocking AAA3 ATP hydrolysis does not block dynein motility in vivo if the dynactin complex is present, (2) Intriguingly, while dynactin and cargo adapter are both needed for dynein activation in wild-type cells and cells with the phi-opening mutations, blocking AAA3 ATP hydrolysis allows the requirement for cargo adapter to be bypassed, which causes dynein to move prematurely without carrying its cargo in vivo, (3) Presence of ATP in AAA3 may possibly promote phi-opening, causing the need for LIS1 to be bypassed.

P1894/B134

Dynein Adaptors Strategically Couple ER Membrane Penetration and Disassembly of a Viral Cargo.

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During entry, viruses must navigate through the host endomembrane system, penetrates cellular membranes, and undergo capsid disassembly to reach an intracellular destination that supports infection. How these events are coordinated is unclear. Here we reveal an unexpected function of a cellular motor adaptor that coordinates virus membrane penetration and disassembly. Polyomavirus SV40 traffics to the endoplasmic reticulum (ER) and penetrates a virus-induced structure in the ER membrane called focus to reach the cytosol where it disassembles prior to nuclear entry to promote infection. We now demonstrate that the ER focus is constructed proximal to the Golgi-associated BICD2 and BICDR1 dynein motor adaptors; this juxtaposition enables the adaptors to directly bind to and disassemble SV40 upon arrival to the cytosol. Our findings demonstrate that strategic positioning of the

virus membrane penetration site couples two decisive infection events — cytosol arrival and disassembly — and suggest cargo-remodeling as a novel function of dynein adaptors.

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Microtubule Dynamics and its Regulation 3

P1895/B136

Clasp Mediates Microtubule Repair by Promoting Tubulin Incorporation into Damaged Lattices.

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Microtubule network in cells plays a key role in processes like cell division, motility and intracellular trafficking. It is subjected to various mechanical stresses induced by acto-myosin contractility, motor based organelle transport and morphological changes during cell motility. Microtubule lattices have always been seen as relatively stable structures that mostly undergo turnover upon depolymerization from the plus ends due to dynamic instability. However recent evidence suggests that microtubules exchange tubulin dimers at the sites of lattice defects, which can either be induced by mechanical stress or occur spontaneously during polymerization. Tubulin incorporation restores microtubule integrity; moreover, "islands" of freshly incorporated, GTP-bound tubulin can slow down microtubule disassembly and promote rescues. Microtubule repair can occur in vitro in the presence of purified tubulin. However, in cells it is likely to be regulated by specific factors, the nature of which is currently unknown. CLASP is a known microtubule-stabilizing factor, which suppresses catastrophes and induces microtubule nucleation and rescue. Since CLASP promotes recovery of tapered growing plus ends with lagging protofilaments into complete ones, it is an interesting potential candidate to promote microtubule repair. Here, we used in vitro reconstitution assays combined with microfluidics and laser microsurgery to show that CLASP2α indeed stimulates microtubule lattice repair. CLASP2α promotes tubulin incorporation into damaged lattice sites thereby restoring the integrity of the tube. Furthermore, it induces the formation of complete tubes from partial protofilament assemblies and restores the stiffness of microtubules softened by hydrodynamic flow. A single CLASP2α domain, TOG2, which suppresses catastrophes when tethered to microtubules, was sufficient to stimulate microtubule repair, indicating that catastrophe suppression and lattice repair are mechanistically similar. Our results suggest that the cellular machinery controlling microtubule nucleation and growth also protects microtubules against physiological insults.

P1896/B137

Severing Enzymes Amplify Microtubule Arrays through Lattice GTP-tubulin Incorporation.

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Spastin and katanin sever and destabilize microtubules. Their function is important in basic cellular processes ranging from cell division to neurogenesis and phototropism. Paradoxically, despite their destructive activity they increase microtubule mass in cells. We combined single-molecule total internal reflection fluorescence microscopy and electron microscopy to show that the elemental step in microtubule severing is the generation of nanoscale damage throughout the microtubule by active ATP hydrolysis-dependent extraction of tubulin heterodimers. These damage sites are repaired spontaneously by guanosine triphosphate (GTP)–tubulin incorporation, which rejuvenates and stabilizes the microtubule shaft. Consequently, spastin and katanin increase microtubule rescue rates. The incorporation of GTP-tubulin into the microtubule shaft is directly responsible for the increase in rescue rates, as the same increase is not elicited by either enzyme in the presence of non-hydrolyzable ATP analog. These GTP-tubulin “islands” transiently recruit end binding protein 1 (EB1) and rescues occur preferentially at these GTP-tubulin islands. Furthermore, newly severed ends of the microtubule emerge with a high density of GTP-tubulin that protects them against spontaneous depolymerization. The stabilization of the newly severed plus ends and the higher rescue frequency synergize to amplify microtubule number and mass. Thus, severing enzymes regulate microtubule architecture and dynamics by promoting GTP-tubulin incorporation within the microtubule shaft. This microtubule-based amplification mechanism in the absence of a nucleating factor helps explain why the loss of spastin and katanin results in the loss of microtubule mass in systems that are dependent on noncentrosomal microtubule generation.

P1897/B138

Does Dendrite Identity Depend on the Presence of Minus-end-out Microtubules?

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Microtubules (MTs) are important for the development and maintenance of neurons. MTs are intrinsically polarized polymers with dynamic plus-ends and less dynamic minus-ends. In mammals, axons have uniform plus-end-out MTs while dendrites typically contain a significant proportion of minus-end-out MTs. Previous in-vitro studies suggest that loss of minus-end-out MTs causes dendrites to gain axonal features. The objective of this study is to determine if loss of minus-end-out MTs can affect dendrite identity in-vivo. We have developed a method in Drosophila neurons that eliminates minus-end-out MTs from certain regions of the dendritic arbor, leading to a subset of neurites with flipped polarity resembling axons. Using this method, we studied the effect of flipped polarity of MTs on different neuronal properties. We did not observe a significant difference in morphology between the minus-end-out and flipped neurites when we compared their length and number of branches. In contrast, MT stability and cargo distribution was affected by MT polarity. The average number of MT plus ends per unit length in the flipped neurites was about half the number in minus-end-out neurites, mirroring the increased MT stability of axons compared to dendrites. Neuropeptide-containing synaptic vesicles concentrated at tips of mixed or flipped neurites, but not minus-end-out neurites, suggesting that these axonal vesicles are distributed based on MT polarity. We also found fewer ribosomes

(dendritic marker) in flipped neurites compared to minus-end-out neurites. These data together suggest that the change in polarity can affect MT stability and localization of compartment-specific cargo, but, surprisingly, not overall shape. We further probed identity of flipped neurites by injuring them. After a dendrite is severed from the cell body, MT dynamics is increased in the cut-off piece. While minus-end-out regions of the arbor showed this response, the flipped neurites did not. All these properties together suggest that neurites with flipped MT polarity tend to lose dendritic features and gain axonal features. This study strengthens the view that MT pattern underlies neuronal polarity and particularly highlights the significance of minus-end-out MTs in defining dendrite identity.

P1898/B139

Rac1 Promotes Septin-mediated Guidance of Non-centrosomal Microtubules to Focal Adhesions.

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During angiogenesis, endothelial cells (ECs) utilize signaling molecules and microtubule (MT) associated proteins (MAPs) to control the dynamic and coordinated remodeling of the actin and MT cytoskeleton to become polarized. Rac1, a signaling molecule of the Rho GTPase family, promotes cell protrusion and enhances MT plus-end assembly into protrusions, thereby driving EC polarization. As ECs polarize, actin filaments assemble and become linked to focal adhesion (FA) complexes that mature at the positions where the cell interfaces with the extracellular matrix. Recent investigations have identified two distinct populations of MTs, centrosomal and non-centrosomal. Loss of centrosomal MTs had no effect on EC polarization, while non-centrosomal MTs were found to be required for EC sprouting and directed migration. Despite this discovery, how ECs distinguish different populations of MTs to drive fundamental cell functions remains unclear. Non-centrosomal MT minus-ends are known to associate with MAPs that inhibit MT disassembly, and promote plus-end MT growth. Septins are MAPs that promote actin stress fiber-mediated maturation of FAs and that spatially guide MT plus-end dynamics. Collectively, these data point to a potential mechanism used by ECs to delineate MT functions via association with septins. Here, we tested the hypothesis that Rac1 activates septin-mediated guidance of non-centrosomal MTs to promote MT growth into FAs. Live-cell fluorescence imaging of ECs revealed that MTs that penetrate FAs are predominantly non-centrosomal. Optogenetic activation of Rac1 promoted septin re-localization adjacent to peripheral FAs. Moreover, Rac1 activation prevented septin re-localization even after pharmacologic inhibition of myosin II. NC MTs consistently grew into peripheral septin resulting in growth of MTs into FAs and reduction in FA size. Lastly, activation of the MT depolymerizer MCAK reveals increased interaction with septin and FAs but inactivation of MCAK increased FA size. Taken together, these data suggest localized Rac1 activity drives the peripheral localization of septins that subsequently guides NC MT growth into FAs to promote FA disassembly. Moreover, differential regulation of MCAK activity on NC MTs aids septin targeting and growth into FAs. Future studies will further delve into the contribution of MCAK on FA targeting and to further understand MCAK activation in relation to FA turnover. Morevoer, since myosin II inhibitiom affects septin localization and MT targeting to FAs, septin recruitment of MTs will be analyzed with reference to actin stress fibers.

P1899/B140

A Clip-170-induced +Tip Network Superstructure Has Characteristics in Cells Consistent with a Liquid Condensate.

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Proper regulation of microtubule (MT) dynamics is critical for many cellular processes such as cell division and intracellular transport. Microtubule plus-end tracking proteins (+TIPs) dynamically track growing MT tips and play a key role in this regulation. +TIPs interact with each other in a complex web of intra- and inter- molecular interactions known as the +TIP network. Explanations for the purpose of the +TIP:+TIP interactions include localizing MT regulators to growing MT tip and relieving their autoinhibition. Our group is interested in the possibility that the +TIP network also has a physical function: we suggest that it creates a dynamic assembly that surrounds the fragile MT tip, constraining its structural fluctuations and therefore promoting MT assembly (Gupta, Bioessays. 2014). The observation that many +TIP network proteins are multivalent and contain intrinsically disordered regions suggests that the +TIP network as assembled on MT tips might constitute a liquid condensate (also known as liquid droplet or membraneless organelle). Such a condensate could potentially form a sleeve-like structure at the tip, providing an attractive model for how the +TIP network might physically promote MT polymerization. It is difficult to test this hypothesis directly. However, previous studies have shown that overexpression of the +TIP CLIP-170 induces large structures that do not co-localize with membrane markers but do contain CLIP-170 and other members of the +TIP network. We hypothesize that these overexpression-induced structures ("patches") might reflect the biological properties of the endogenous +TIP network, and so we are investigating whether they have the properties predicted for liquid condensates. Video microscopy experiments show that the GFP-CLIP-170 induced condensates can elastically deform and undergo fission and fusion. Fluorescence Recovery After Photobleaching (FRAP) experiments demonstrate that CLIP-170 dynamically exchanges both within a patch and between a patch and the cytoplasm. These results indicate that the patches have properties consistent with liquid condensates and are not simply protein aggregates. Immunofluescence experiments show that the patches contain a range of +TIP network proteins but exclude molecules found in other liquid condensates. Taken together, these results indicate that the CLIP-170 induced patches in cells are phase-separated liquid condensates consisting of +TIP network proteins, and they suggest that the endogenous +TIP network might also form liquid droplets at MT tips.

P1900/B141

Uncovering Microtubule-driven Mechanisms of Melanoma Invasion.

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Metastatic melanoma is currently incurable and available therapies, although effective, result in resistance and recurrence. The majority of deaths are due to metastatic disease, highlighting the need for ‘migrastatics’, therapeutics which act to inhibit invasion. A recent paradigm shift positions the extracellular matrix as a key player in the metastatic cascade. Cell navigation of 3D matrix requires adaptive changes in cell and nuclear shape to fit matrix physical attributes in a process termed mechanosensing. This process incorporates dynamic remodelling of cell matrix adhesions and the cytoskeleton, to facilitate movement through confined spaces, via proteolytic matrix degradation or cell

squeezing. Microtubules play a pivotal role in both of these processes. Our data show that the microtubule-binding proteins, CLASPs, are highly overexpressed in metastatic melanoma lines where they regulate the resistance of microtubule mechanical compression during melanoma invasion in 3D collagen matrices. Using high-resolution live-cell microscopy coupled to genetic alteration and substrate microfabrication, we have identified that patient-derived Melanoma cells utilise CLASP1 and CLASP2, for differing functions to drive 3D invasion. We report paralog specific depletion of CLASPs results in strikingly different 3D invasion phenotypes. Crucially, paralog specific depletion of CLASPs ablates the ability to inter-convert between adaptive invasion strategies by interfering with microtubule-dependent functions during 3D-invasion. Furthermore, pan-depletion of CLASPs within 1205Lu melanoma cells results in 3D migration stasis and reduced cell viability following conditions of 3D confinement, which we do not observe in 2D. These findings suggest that CLASPs function in melanoma cells to facilitate biomechanically regulated cellular processes of both invasion and survival in confined environments.

P1901/B142

Effect of Cytoplasm Concentration on Cytoskeleton Dynamics.

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The cytoplasm is a very crowded environment harboring most of the metabolic reactions necessary for cellular function. Its biophysical properties are influenced by cellular metabolism. Indeed, changes in cytoplasm mechanical properties have been linked to cellular differentiation and changes in cytoplasm concentration to cell death. However, the reverse relationship has not been extensively studied. So, here we tried to address the effects of the cytoplasm biophysical properties on various dynamic processes happening inside cells from protein diffusion to cytoskeleton dynamics. Our results demonstrate the sensitivity of cellular dynamics, specifically of the cytoskeleton dynamics, to cytoplasm concentration. In fission yeast, changing cytoplasmic concentration reversibly affects all the dynamic processes we looked at from protein diffusion to complex dynamic processes such as actin and microtubule dynamics. We found that microtubule dynamics is inversely correlated to cytoplasmic concentration. Surprisingly, cytoplasmic concentration not only slowed down the microtubule growth rate but also the shrinkage rate. Moreover, the effect of cytoplasmic concentration on cytoskeleton dynamics did not seem to depend on stress pathway. We extended our observation to other eukaryotes, HeLa cells and moss, and confirmed the effect of cytoplasmic concentration on microtubule dynamics suggesting that we are probing an inherent property of the cytoplasm. Our results highlight how important the biophysical properties of the cytoplasm are to cellular metabolism in eukaryotes. This study reveals the interplay between biology and physics happening in the cytoplasm. Our assay is a first step in understanding how the biophysical properties of the cellular environment affect the biological processes it encloses.

P1902/B143

A Pushing Mechanism for Microtubule Aster Positioning in a Large Cell Type.

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Following fertilization, microtubule (MT) sperm asters accurately position male and female pronuclei allowing for pronuclear fusion, a process essential for dictating the first division plane. Due to the relatively large sizes of zygotes compared to somatic cells, the forces that drive sperm aster migration

have been hypothesized to be due to pulling on the leading astral MTs by retrograde motility of dynein anchored either to the cell cortex or to cytoplasmic vesicles. Conversely, pushing forces from rear facing MTs growing against the cortex are have not been documented due to potential MT buckling and slipping as they grow to greater lengths. Here, we re-investigate the forces required for sperm aster centration in the large sea urchin zygote (~100 um diameter) using live confocal microscopy of astral MTs and MT plus-ends, chemical manipulation of aster sizes, inhibition of dynein, and targeted ablation of the sperm aster. We find that leading, front portions of the aster are shorter and less dense than rear, cortical facing portions of the aster. This aster geometry precludes the current cytoplasmic pulling model, which is dependent on front portions of the aster that are longer and more dense than the rear portions of the aster. Second, we find that aster migration distance and rates are equal to and limited by the length and growth rates of rear portions of the aster, with no influence from front lengths and growth rates. Rear astral MTs are also anchored to the cortex, which would antagonize pulling from dynein along front astral MTs. Ciliobrevin treatment, which was previously reported to inhibit aster migration in this system, causes immediate disruption of sperm aster integrity, complicating interpretations of ciliobrevin as a probe for dynein function. Therefore we inhibit dynein by injecting the CC1 subunit of p150 and follow aster migration. CC1 injection does not abrogate sperm aster migration despite inhibiting normal mitotic spindle formation indicating that dynein is not essential for aster migration in the sea urchin zygote. Finally, using the UV light inducible MT depolymerizing agent, caged-combretastatin, we show that ablation of rear astral MTs halts aster migration, while ablations of front and side astral MTs does not block aster migration. Collectively, our data indicates that a pushing mechanism by rear, cortical facing MTs can drive aster migration in a large cell type.

P1903/B144

Multi-component in *Vitro* Reconstitution Induces Robust Microtubule Treadmilling.

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Dynamic instability, in which a microtubule end switches between phases of growth and shrinkage, is the hallmark behavior of microtubules. Microtubule treadmilling, in which the plus end grows while the minus end shrinks, is also observed in cells when minus ends are detached from nucleating centers. While microtubule dynamic instability has been widely studied in *vitro*, the conditions that lead to microtubule treadmilling are not well understood. To elucidate the mechanisms underlying microtubule treadmilling, we first characterized microtubule dynamics in *vitro* with purified tubulin over a range of tubulin concentrations, and analytically calculated a net assembly/disassembly rate at both polymer ends. Our population-level results predicted that microtubules assembled with tubulin alone can treadmill, albeit with minus ends leading, consistent with earlier reports. We then used an in *vitro* assay that allows observation of treadmilling on a single-microtubule level. In conditions predicted for treadmilling, we observed a significant fraction of microtubules that could be classified as treadmilling, with the majority exhibiting minus-end directionality. However, not only was the treadmilling direction different from that observed in cells, the overall flux rates of these microtubules were an order of magnitude smaller than measured cellular rates. We hypothesized that this discrepancy is due to the regulatory effects of Microtubule Associated Proteins (MAPs) in cellular environments. To test this hypothesis, we explored the combined effects of MAPs on microtubule assembly flux rates using computer simulations, constrained by published experimental observations. Our in *silico* experiments predicted that a combination of four MAPs (EB1, XMAP215, CLASP2 and MCAK) could promote plus-end-leading treadmilling with high flux rates. Finally, we tested the predictions of our computational model

using the multi-MAP in *vitro* microtubule assembly assay, and found that with this minimal in *vitro* system, we could indeed reconstitute robust and fast plus-end-leading treadmilling, consistent with observations in cells.

P1904/B145

The Effect of Tubulin Arginylation on Cellular Microtubules.

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Microtubules are involved in a host of cellular processes required for development and health, including cell division, cell morphology, cell motility, and intracellular transport. These functions require morphologically and dynamically different microtubule populations, and this diversity is often conferred by post-translational modifications. Different tubulin modifications are enriched in specific microtubule-based structures and can regulate the dynamics, stability, and protein interactions of the microtubules they decorate. One tubulin modification that has been identified but not well studied is arginylation. Arginylation is the post-translational addition of arginine to glutamate and/or aspartate residues within a protein by arginyl transfer enzyme 1 (ATE1). In mice, *Ate1* deletion causes abnormal cardiac and neurological development, resulting in embryonic lethality starting at E12.5. Many of the observed phenotypes appear to be the result of impaired cytoskeletal function. Accordingly, cellular studies have shown that *Ate1* deletion causes cytoskeletal defects, including reduced cell motility and adhesion. These processes are known to involve microtubules, but the role of tubulin in these defects has not yet been explored. Previously, tubulin arginylation has been identified by mass spectrometry of whole mouse embryos and by in *vitro* arginylation reactions. Here, mass spectrometry of taxol purified microtubules from mouse embryonic fibroblasts (MEFs) has identified arginylation on several residues in both α- and β-tubulin, in regions that have the potential to alter electrostatic interactions and thus to regulate microtubule dynamics and/or stability. MEFs with *Ate1* knockout (*Ate1*$^{-/-}$) show a significant reduction in end binding 1 (EB1) comet velocity compared to wildtype MEFs, suggesting that lack of arginylation results in slower microtubule growth rate. Additionally, *Ate1*$^{-/-}$ MEFs show an increased fraction of depolymerization resistant microtubules compared to wildtype, suggesting that lack of arginylation results in an increased population of stable microtubules. Together these results demonstrate that arginylation plays a role in regulating the dynamics and stability of cellular microtubules.

P1905/B146

Kinetochore Proteins Function Post-mitotically to Influence Dendrite Regeneration and the Neuronal Cytoskeleton.

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The kinetochore is a complex of highly conserved proteins that is responsible for proper chromosome segregation during mitosis. Until recently there has not been reason to study these proteins outside of cell division, much less in post-mitotic neurons. By comparing RNAseq data from control and injured neurons, we found that a set of kinetochore genes was upregulated 6 hours after dendrite injury but not after axotomy in Drosophila ddaC neurons. We subsequently discovered that knocking down some of these proteins post-mitotically with RNAi in *vivo* caused a deficit in dendrite regeneration without impairing axon regeneration. However, normal morphology of these cells was not impaired with these knockdowns. To understand how kinetochore proteins might impact dendrite regeneration, we

examined microtubule dynamics in neurons, as the kinetochore is the link between microtubules and chromatin. In neurons with kinetochore components knocked down, microtubule dynamics in axons was normal but in dendrites was enhanced. This phenotype of more dynamic microtubules was present in dendrites of two neuron types following kinetochore protein knockdown. The enhanced microtubule dynamics was independent of cell stress, as it was not rescued by a JNK inhibitor. We conclude that a set of kinetochore proteins is repurposed in post-mitotic neurons to modulate the microtubule cytoskeleton in dendrites as well as promote dendrite regeneration.

P1906/B147

Regulation of Kinetochore Function by the Ras/pka Pathway in *Saccharomyces Cerevisiae*.

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Faithful chromosome segregation during cell division over generations is required for the health and survival of organisms. This process is mediated by two major events: (1) stable attachments between the microtubules (MTs) nucleated from the spindle pole body (SPB) and sister chromatid kinetochores (KTs) assembled at the centromeres; (2) partitioning of sister chromatids to daughter cells. Dam1C and Ndc80C are the two major KT complexes that physically bind to the incoming MT. The heterodecameric Dam1 complex (Dam1C) is an efficient microtubule stabilizer and forms the outermost part of a kinetochore. Oligomers of Dam1c form rings that embrace the depolymerizing plus ends of MT's during anaphase to ensure chromosomes remain stably bound as they move to the opposite spindle poles. We previously reported for the first time that PKA directly acts on KT function and chromosome segregation by phosphorylating Dam1 at S31, a subunit of the Dam1C. However, the function of Dam1 phosphorylation by PKA remains unclear. It is well established that the Aurora B kinase (Ipl1p in yeast) is a major regulator of MT-KT attachments by phosphorylating multiple KT subunits. Interestingly, we found that phosphomimetic *dam1S31D* rescues the temperature sensitivity (ts) of *ipl1* mutants. Thus, PKA may work together with Ipl1 in modulating MT-KT attachments. Furthermore, cell imaging experiments revealed that phosphorylation of S31 contributes to resolution of aberrant attachments that occur prior to entering metaphase. We are working to determine how phosphorylation of S31 influences KT-MT dissociation and to identify additional KT subunits that are PKA targets. Our new work will help to build a better understanding of how PKA functions in chromosome segregation and provides a model for how extrinsic factors such as nutrients can modulate chromosome segregation.

P1907/B148

A Role of DRG2 in Brain Cell and a Mouse Model of the Alzheimer Disease.

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Our previous study indicated abnormal behaviors of microtubule dynamics and phosphorylation of tau S202 in the developmentally regulated GTP binding protein 2(DRG2) KD HeLa cells. Alzheimer disease (AD) is characterized to be "tauopathies" by the abnormally phosphorylated tau protein. Here, we initiate to find a link between DRG2 and AD. The depletion of DRG2 in BV-2 microglial cell line, which is associated with AD plaques containing β-amyloid (Aβ), leads to decrease Aβ and Fe65(*Apbb1*) expression. The expression of Aβ is down-regulated in the hippocampi of the *DRG2* KO mice. We also show that DRG2 expression was increased in the hippocampi from streptozotocin-induced Alzheimer

animal model. Taken together, our results suggest that DRG2 is associated with deposition of Aβ in AD. (Supported by the Grant NRF-2017R1D1A3B04030339 of Korea)

P1908/B149

Dynamics of Meiotic Spindles in Mouse Oocytes.

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In mouse oocytes, meiosis arrests in metaphase II for up to 12 hours, awaiting the arrival of a sperm to trigger anaphase. While the morphology and global properties of the meiotic spindle are maintained during this time, the period of arrest is nevertheless highly dynamic, as individual microtubules continuously polymerize and depolymerize, molecular motors slide microtubules along each other, and microtubule bundles -- locally dense regions -- form and dissolve. Previous work has shown that, for Xenopus laevis spindles reconstituted in vitro, these processes can be described by a coarse-grained continuum model, in which the microtubules that make up the spindle are treated as an (active) liquid crystal. In this poster, we will present the results of our attempts to apply these concepts to the meiotic spindles in living mouse oocytes, and discuss the applicability of continuum theories in this case.

P1909/B150

Computer-based Simulation of the Heterodimer Interface in Microtubules to an alyze the Impact of Phosphorylation of Ser^{165} in Alpha-tubulin.

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Previous studies with human breast cells demonstrated that phosphorylation of alpha-tubulin at Ser^{165} by protein kinase C results in marked changes in microtubule dynamics that included a prolonged elongation phase and increased rate of elongation (*Cytoskeleton* 71:257-272, 2014). As verified with site-direct mutants of alpha-tubulin (S165D, S165N), microtubule growth correlated with dramatically increased cell motility and other hallmarks of the epithelial-mesenchymal transition. Ser^{165} in alpha-tubulin occurs near the heterodimer interface and is within 10Å of the exchangeable GTP on beta-tubulin and Glu^{254}, the catalytic residue (on alpha-tubulin) that hydrolyzes the exchangeable GTP. It is generally believed that the growing end of a microtubule is capped by several units of GTP-bound beta-tubulin. Our hypothesis is that phosphorylation at Ser^{165} (as given by the S165D mutant) perturbs the alignment of Glu^{254} with respect to the exchangeable GTP thereby preventing its hydrolysis and consequently giving rise to persistent polymer growth. In the present study, molecular simulations were performed with cryoEM structures of microtubules (PDB 3J6E) into which a mutation had been introduced at Ser^{165}. The objective was to identify whether the S165D mutant alters the intramolecular distance between the catalytic residue Glu^{254} and bound GTP. The results revealed that S165D produced only a slight decrease in the intermolecular distance. However, the impact by S165D was stronger with respect to the distance between Ser^{165} and the loop-helix defined by Gln^{256}/Ala^{247}; this sub-structure was pulled into the heterodimer cleft and formed a H-bond at the GTP binding site with Gln^{451} in beta-tubulin that suggested weaker GTP binding. These results indicated that phosphorylation at Ser^{165} causes structural alterations at the heterodimer interface. However, further study is required to establish whether these perturbations affect GTP binding and/or hydrolysis.

P1910/B151

Actomoysin Dynamics Modulate Microtubules Deformation and Growth during T Cell Activation.

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The activation of T lymphocytes is an essential step in the adaptive immune response. T cell activation involves the binding of T cell receptors (TCR) with antigen on the surface of antigen presenting cells (APC), which leads to the spreading of the T cell onto the APC and formation of the immunological synapse (IS). Proper formation and maturation of the IS requires the polarization of the T cell and coordination between different cytoskeletal systems. T cell spreading is characterized by remodeling of the actomyosin cytoskeleton and reorientation of the microtubule-organizing center (MTOC) towards the activating surface. Despite extensive research, the interactions between the different cytoskeletal components during T cell activation are not well understood. In this work, we imaged multiple distinct fluorescently labeled cytoskeletal structures in Jurkat T cells using instant structured illumination microscopy (iSIM) and TIRF microscopy. We found that microtubules (MT) filaments were more dynamic at the peripheral actomyosin rich region of the cell-substrate contact compared to the central actin-depleted zone. Microtubules in actin rich zones also displayed more deformed shapes as indicated by their local curvature distributions. We used small molecule inhibitors to explore the role of different cytoskeletal modulators and molecular motors that regulate the forces responsible for the deformations. We found that formin inhibition reduced MT growth rates and reduced the deformation of MT filaments. Inhibition of ROCK kinase using Y27632 resulted in a further reduction of MT deformation, suggesting that actomyosin contractility plays an important role in defining MT shapes. We found that MT tip growth speed is slower when passing through actin rich regions as compared to actin poor regions. These differences disappeared in cells treated with Arp2/3 inhibitor CK666, suggesting that branched actin networks impose a physical barrier to growing microtubules. Our results indicate an important mechanical coupling between the actomyosin and microtubule systems where different actin structures influence microtubule dynamics in distinct ways.

P1911/B152

SSNA1 Stabilizes Microtubule Dynamics in *Vitro*.

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Sjogren's Syndrome Nuclear Autoantigen 1 (SSNA1) is a microtubule-associated protein that has been implicated in cilia assembly, cell division and axonal branching; however, the effect of SSNA1 on microtubule dynamics is not known. We employed in *vitro* reconstitution techniques with purified protein components combined with TIRF microscopy to investigate the effects of human SSNA1 on microtubule dynamics. Using Alexa 488-labeled SSNA1, we show that SSNA1 binds and bundles GMPCPP-stabilized microtubules. Interestingly, when incubated with dynamic microtubules, SSNA1 preferentially localizes to newly-formed microtubule lattices indicating that SSNA1 co-polymerizes with soluble tubulin. Furthermore, we find that SSNA1 suppresses microtubule growth and catastrophe (the transition from growth to shrinkage), and promotes rescue (the transition from shrinkage to growth). To further probe the anti-catastrophe activity of SSNA1, we polymerized dynamic microtubules with or without SSNA1 and then depleted the available soluble tubulin concentration by either dilution or by stathmin-mediated tubulin sequestration; in both cases, SSNA1 was able to robustly protect

microtubules against catastrophe. Our results indicate that SSNA1 is a potent microtubule stabilizer that prevents microtubules from undergoing catastrophe. The microtubule stabilizing activity likely underlies the role of SSNA1 in cilia function, cell division and neuronal development.

P1912/B153

Mammalian CLASP1 and CLASP2 Act in a Functional Hierarchy to Support Microtubule Nucleation, anchoring, and Plus-end Dynamics.

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Microtubule (MT) plus-end tracking proteins CLASPs regulate MT dynamics and nucleation. Mammalian paralogs CLASP1 and CLASP2 are highly similar, however, unique functions of each protein or mutual regulatory interactions have not been studied extensively. Here, we show that CLASP1 and CLASP2 have different functions relating to nucleation and anchoring of non-centrosomal MTs, as well as MT plus end dynamics. Our data indicate that in cells CLASP1 promotes robust localization of CLASP2 to MT plus ends via a direct protein-protein interaction. Accordingly, CLASP2 acts downstream of CLASP1 in those functions that depend on MT plus end positioning, in particular the support of MT nucleation and MT plus end dynamics. Interestingly, we found that while these two functions are related, CLASP1 is more efficient in supporting MT nucleation, while CLASP2 is critical in regulating MT growth rates. At the same time, CLASP2 has a unique cellular location at the Golgi membrane, where CLASP1 is not found. We find that Golgi-associated CLASP2 pool is necessary for anchoring of nascent Golgi-derived MTs (GDMTs) to the Golgi membrane. In summary, our data dissect functional hierarchy between mammalian CLASP paralogs and determines specific functions of these two proteins in MT dynamics and organization.

Tubulins and Associated Proteins 2

P1913/B154

Expression Levels of α- and β-tubulin Reveals Collusion between Subunits.

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The microtubule cytoskeleton undergoes major reorganizations during mitosis, process extension and ciliogenesis. To do this, cells must rapidly increase the supply of tubulin heterodimers, and, accordingly, the levels of α- and β-tubulin protein monomers. Therefore, a fundamental question is how cells control levels of α- and β-tubulin so that the stoichiometry heterodimer is maintained. Previous work has provided insights into how α- and β-tubulin are individually regulated. However, these studies have not addressed whether there is a mechanism that coordinates regulation between α- and β-tubulin subunits to maintain stoichiometry for heterodimer assembly. We address this question using the budding yeast model system, generating tools to control the copy number and expression of α- or β-tubulin genes. We monitor total α- and β-tubulin levels in the cell as well as the amount of heterodimer and observe impacts on microtubule dynamics and function during mitosis. Our findings demonstrate when β-tubulin protein levels are decreased by knocking out one gene copy, α-tubulin protein levels decrease in response. However, β-tubulin protein levels do not respond when α-tubulin is decreased by knocking out one gene copy. Additionally, we provide evidence for toxicity associated with excess β-tubulin in cells. Our results support a model in which the cell only maintains a level of α-tubulin protein that is needed for the appropriate level of β-tubulin and necessary to prevent detrimental effects associated with excess β-tubulin.

P1914/B155

Parthenolide Destabilizes Microtubules by Forming Cysteine Adducts on Tubulin.

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Tubulin is subjected to various post-translational modifications such as acetylation, methylation, phosphorylation, polyglutamylation, and detyrosination. Tubulin detyrosination has been studied for nearly 4 decades, but the carboxypeptidase that targets tubulin (TCP) was only recently identified as vasohibin (VASH) and its associated small vasohibin binding protein (SVBP). Prior to the identification of VASH-SVBP, tubulin detyrosination was studied using broad spectrum carboxypeptidases (*eg.*, carboxypeptidase A) and a sesquiterpene lactone, parthnolide (PTL), that was reported to block tubulin detyrosination in cells. However, PTL exhibits polypharmacology and has been commonly used as an anti-cancer and anti-inflammatory drug through modulation of cytochrome c and nuclear factor-κB activities. We found that PTL treatment induced cell rounding and destabilized microtubules during both mitosis and interphase. However, PTL does not inhibit the tubulin detyrosination activity of VASH1-SVBP in *vitro*. Rather, we demonstrate that PTL affects the biochemical properties of tubulin through a non-specific cysteine-targeting mechanism. First, PTL induces mobility shifts of tubulin during SDS-PAGE. Second, PTL induces tubulin aggregation. Third, mass spectrometry analysis revealed that PTL formed covalent adducts with cysteine residues within both alpha- and beta- tubulin. Importantly, we demonstrate that PTL also forms adducts on tubulin when added to cells in culture. We suggest that PTL induces microtubule destabilization by reducing the polymerization-competent pool of tubulin in cells through adduct formation. Finally, we demonstrate that the recently introduced TCP inhibitor, epoY, is a potent inhibitor of VASH both in *vitro* and in *vivo*. Altogether, our results suggest that PTL targets tubulin through a non-specific mechanism, and we strongly encourage the use of epoY instead of PTL when studying tubulin detyrosination.

P1915/B156

Studying of a Connection between α,β-tubulin Conformations and Microtubule Dynamics.

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Microtubules (MTs) are αβ-tubulin polymers that are essential for chromosome segregation and intracellular organization. MTs are always in a state of dynamic instability, which means MTs constantly switch between growing and shrinking phases. The dynamic properties of MTs essential for their function. However, the molecular mechanisms underlying microtubule dynamics are incompletely understood. αβ-tubulin cycles through different conformations during MT growth and shrinking. Individual αβ-tubulins display a 'curved' conformation, but adopt different 'straight' conformations after binding to MT end. We are studying the relationship between the αβ-tubulin conformation cycle and microtubule dynamics using site-directed mutagenesis of αβ-tubulin. The core helices of α- and β-tubulin are positioned differently during the conformational cycle. These helices may operate as a structural relay to transmit information from one end of the fold to the other. In a prior study, we showed that a T238A mutation at the bottom of the core helix of β-tubulin reduced catastrophe frequency and MT shrinking rate, with little effect on growth rate. We were not able to determine if a similar mutation in α-tubulin yielded similar effects, because the muted site is not conserved between α and β tubulin. As a next step, we want to study mutation at positions that equivalently buried in both α and β tubulin. We a targeting a cluster of residues that are positioned around the core helix and that are

conserved between α - and β - tubulin. We will present an update on our progress. Ultimately, these studies are likely to provide new insights into the role of the ab-tubulin conformation cycle and whether both α- and β- tubulin contribute comparably.

P1916/B157

Binding Affinity of Tau Repeat Regions with Neuronal Specific β-tubulin Isotypes.

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Tau is a microtubule-associated protein which is abundantly expressed in neurons. Tau plays an important role in the assembly and stabilization of microtubules which are formed by polymerization of α and β tubulin subunits. The C-terminal domain of Tau consists of four repeat regions R1, R2, R3 and R4 which bind to microtubules to stabilize them. It is known that the detachment of tau from the microtubules leads to the formation of cytoplasmic insoluble aggregates which forms the basis of various neurodegenerative diseases. Microtubules are made up of αβ tubulin subunits. Seven α-tubulin and nine β-tubulin isotypes have been reported to be present in humans till date. These tubulin isotypes show residue composition variations mainly at C-terminal region and bind to motor proteins and anti-mitotic drugs differently. These tubulin isotypes show tissue specific expression as their relative proportion varies significantly in different type of cells. These isotypes show differential microtubule dynamics as well as differential binding affinity towards anti-mitotic drugs and motor proteins. However, the relative binding affinities of tau repeat regions with neuronal-specific tubulin isotypes are not completely known due to lack of their structural information. Using a combination of molecular modelling, molecular docking and molecular dynamics simulations we have explored the relative binding affinity of tau repeat regions with neuronal-specific tubulin isotypes.

P1917/B158

Regulation of Tpx2 by Importins-ab.

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The microtubule based mitotic spindle is responsible for equally partitioning the genome with each cell division. This process is enabled by equal rapid and accurate spindle assembly, which begins with MT nucleation. Targeting Protein for XKlp2 (TPX2) promotes MT nucleation around chromosomes and promotes branching MT nucleation, where MTs form on the lattice of a pre-existing MTs. TPX2 is regulated by importins-α/β, but the molecular nature of this regulation remains unclear. Here we demonstrate that TPX2 interacts with importins-α/β with nanomolar affinity as a 1:1:1 mono-dispersed trimer. Furthermore, importin-α mediates this high affinity interaction and does so via one of two nuclear localization sequences (NLS) - one of which (at aa123-126) was previously not annotated. Interestingly, both importin-α and importin-β associate with TPX2 via dispersed, weak, Van der Waals interactions. These interactions appear to be the molecular driving forces of importin-mediated inhibition of TPX2 phase separation. Lastly inhibition of phase separation is correlated with loss TPX2-mediated branching microtubule nucleation. Collectively our study elucidates the molecular architecture of a complex essential for cell division, provides insight into molecular forces of phase separation, and supports an underappreciated mechanism of regulation involving weak interactions.

P1918/B159

Interactions between Ca^{2+}& Microtubule-actin Crosslinking Factor 1 (MACF1) in Cytoskeletal Remodeling & Cell Adhesion Dynamics.

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Microtubule-actin crosslinking factor 1 (MACF1) is a cytoskeletal crosslinking protein that interacts with F-actin and microtubules. Although there are two putative Ca^{2+}-binding EF hands at its C-terminus, whether and how Ca^{2+} interacts with MACF1 remains elusive. We thus explored how intracellular and extracellular Ca^{2+} regulated cytoskeletal remodeling and cell adhesion respectively. In the intracellular aspect, we studied how Ca^{2+} regulated MACF1-microtubule interaction using GFP-tagged & N-terminus truncated MACF1 proteins with various mutations or deletions on their EF hands. These proteins revealed distinct affinities to microtubules and also to each other in different Ca^{2+} concentrations. We are currently using fluorescent live-cell imaging to investigate their underlying molecular mechanisms. In the extracellular part, our scratch wound-healing assays demonstrated that MACF1 knockdown in cancer cells increased cell-cell coordination in addition to their speed reduction. Together with literature reports that MACF1 knockdown reduced focal adhesion dynamics while increasing levels of adherens junctions & tight junctions-related proteins, we hypothesized that MACF1 induced epithelial-mesenchymal transition (EMT) via directly switching the balance from cell-cell adhesion to cell-matrix adhesion. To verify this hypothesis, we are currently using EGTA to remove extracellular Ca^{2+} and induce E-cadherin internalization to examine how MACF1 alters the dynamics of cell-cell adhesion. Finally, because of the huge size of MACF1 (~600KD), it is not feasible to over-express MACF1. Thus we will use CRISPR to label the endogenous MACF1 molecule with fluorescent proteins, so its dynamic regulation on cytoskeletons and adhesion molecules could be delineated in temporal and spatial manners. With these approaches we will elucidate how MACF1 regulate cytoskeletons and cell adhesion, with the hope to develop novel therapeutic strategies against EMT- and/or adhesion-related diseases in the near future.

P1919/B160

Coordination of the Ndc80 and Ska Complexes at the Human Kinetochore-microtubule Interface.

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Successful segregation of chromosomes during mitosis requires the formation of stable, force-transducing attachments between kinetochores and spindle microtubules. Equally importantly, cells must regulate the strength of kinetochore-microtubule interactions in order to correct erroneous attachments that would lead to chromosome mis-segregation. The kinetochore-associated NDC80 complex is the hub of the kinetochore's microtubule binding activity, and its Hec1/Ndc80 subunit harbors the key microtubule binding site in its globular calponin-homology domain. Hec1/Ndc80 is also a major contributor to attachment strength regulation: its N-terminal "tail" domain is a target for Aurora kinase phosphorylation to promote "release" of incorrect attachments, and dephosphorylation by mitotic phosphatases to strengthen attachments. In addition to the NDC80 complex, the spindle and kinetochore-associated (Ska) complex, comprised of Ska1, Ska2, and Ska3, is a critical factor in stabilizing attachments. How the Ska complex is recruited to kinetochores, and what domain(s) of the NDC80 complex are required for these factors to stabilize kinetochore-microtubule attachments in cells, remain open questions. Here, we investigate how Ska is recruited to kinetochores and which domains of the

NDC80 complex are required for Ska to enhance NDC80-microtubule binding. Using a combination of in *vitro* and in-cell techniques, we find that the Ndc80/Hec1 tail domain is dispensable for kinetochore recruitment of the Ska complex, as well as for Ska-mediated enhancement of NDC80-microtubule binding. In contrast, we find that the Hec1/Ndc80 coiled-coil/loop domain is required for these functions, and all components of the Ska complex localize along the NDC80 complex central coiled-coil domain in cells. Furthermore, we find that the phospho-state of the Hec1/Ndc80 tail has no effect on Ska recruitment to kinetochores, and that kinetochore-microtubule attachments are robustly formed independently of the Ska complex in cells that are blocked of Hec1/Ndc80 tail phosphorylation. However, we demonstrate that the Hec1/Ndc80 tail is not explicitly required for kinetochore-microtubule attachments, but is required for proper spindle maintenance and force generation at the kinetochore, offering a more unified view of Hec1/Ndc80 tail function across organisms.

P1920/B161

Distribution of Posttranslational Modifications Dy/dc2 of A-tubulin C-terminal Tail during Mitosis.

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Microtubules (MTs) are a major cytoskeletal polymer critical for a broad range of cellular processes including cell division, cell migration, and intracellular trafficking in eukaryotic cells. MTs are polymerized from the protein tubulin, a heterodimer of the proteins α-and β-tubulin. However, heterogenous MT populations can be generated inside cells by incorporation of various isotypes, and post-translational modifications (PTMs). How MTs can achieve functional diversity is not fully understood, but one hypothesis is that the ability to carry out diverse functions is enhanced by PTMs. We are specifically focused on a modification of the C-terminal tail of α-tubulin (α-CTT), where the C-terminal tyrosine (Y) is cleaved by VASH1-SVBP leaving the α-CTT detyrosinated (dY). dY-MTs can be further process to dC2 by an unknown carboxy peptidase, and this proteolysis event is thought to be irreversible. This modification is reversed by Tubulin Tyrosine Ligase (TTL) adding tyrosine to the CTT. Deregulation of dY-Y cycle has been shown to impact tumorigenesis and neuro-development. To study the function of TTL, we deleted *TTL* in HeLa cells by genome editing. By immunoblot analysis, clonal isolate 13 (dTTL13) lacks TTL detected and shows enrichment of dY-MTs during all cell cycle phases. Sequencing of the targeted exon 2 identified InDels at three loci introduced by CRISPR/Cas9 gene editing. Because dY-MTs are believed to be more stable than Y-MTs, we examined mitotic progression, a process that is impeded by overly stable MTs, in dTTL13 cells. To examine mitotic progression, we determined the percent mitotic errors, defined as mis/unattached chromosomes during metaphase, and lagging chromosomes in anaphase, for both WT and dTTL13 HeLa cells. dTTL13 shows increased micronuclei relative to WT HeLa, as a result of mitotic errors that coincide with increased dY and dC2-MTs. To further examine the correlation between increased dY-MTs, hyperstable MTs, and chromosome segregation, we challenged dTTL13 cells with sub-lethal doses of taxol, a MT-stabilizing drug. We observed that treatment of dTTL13 cells with taxol increased mitotic errors of dTTL13, suggesting that dY-MTs impair chromosome segregation by elevating MT stability. Current efforts are directed towards defining how dY-MTs prevent accurate chromosome segregation.

P1921/B162

A Structural and Mechanistic Model for the Regulation of LRRK2'S Interaction with Microtubules.

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Parkinson's Disease (PD) is the second most prevalent neurodegenerative disease, affecting ~10 million people worldwide. One of the most commonly mutated genes in PD is **L**eucine **R**ich **R**epeat **K**inase **2** (*LRRK2*). Autosomal dominant mutations in *LRRK2* cause familial PD, while *LRRK2* mutations are also associated with sporadic PD. LRRK2 is a large protein with multiple domains, including a kinase and GTPase domain. A unifying theme for *LRRK2* and many other PD genes is that they function in intracellular trafficking. For example, LRRK2 co-localizes with microtubules, an association that is enhanced by the majority of familial PD mutations and has been shown to disrupt trafficking in model systems. We set out to determine the structure of LRRK2, how LRRK2 interacts with microtubules, and why interactions with microtubules could be detrimental. Using pure components in an in vitro reconstituted system we show that LRRK2 directly binds microtubules. Furthermore, low nanomolar concentrations of LRRK2 inhibit the motility of the microtubule-based motor kinesin-1. Using cryo-electron microscopy, we solved a 3.5Å structure of the catalytic half of LRRK2, which contains its kinase and GTPase domains. Using cryo-electron tomography we solved an 18Å structure of the microtubule-associated filaments formed by LRRK2 in cells. Based on these structures, we built an atomic model of microtubule-associated LRRK2. This model led us to hypothesize that the conformational state of LRRK2's kinase domain is critical for filament formation on microtubules. In support of this model, we find that kinase inhibitors favoring a closed-kinase conformation negatively impact kinesin motility, while kinase inhibitors favoring an open-kinase confirmation relieve the inhibition of kinesin motility caused by LRRK2. In cells, we observe similar trends; with kinase inhibitors that favor an open conformation reducing LRRK2 microtubule-associated filament formation and those favoring a closed conformation enhancing LRRK2 filaments formation. We also find that LRRK2's kinase activity towards the GTPase Rab10 is stimulated by microtubules in vitro. Together, our work leads us to propose that PD-linked LRRK2 uses microtubules as an intracellular scaffold to act as a roadblock for molecular motors and phosphorylate Rab proteins.

P1922/B163

Structural and Functional an alysis of EB1 as a Novel RNA Binding Protein.

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RNA binding proteins (RBPs) play crucial roles in regulating almost all aspects of the RNA life cycle in a cell. Many RBPs have been identified over the years that have a modular organization and comprise a set of canonical RNA binding domains. Recently, high throughput studies have led to the identification of a large number of proteins with putative RNA binding activity in *Drosophila* embryos, despite lacking a canonical RNA binding motif (Sysoev et al., 2016; Wessels et al., 2016). Using a structural-functional approach, we are studying the RNA binding activity of some of these newly discovered RBPs, with the aim of identifying their mechanism of RNA binding and its physiological significance in the cell. Initially, we selected a number of factors known to be involved in cytoskeletal regulation or cell cycle, or to have a specific subcellular localization. One of the candidates we found to interact with RNA in *vitro* is EB1, a master regulator of microtubule plus-end dynamics. In *vitro* assays such as EMSA and NMR titrations performed with EB1 and RNA oligoA and oligoU showed a positive interaction between the protein and the RNAs, and allowed us to map the RNA-protein interaction surface to the microtubule-binding

surface and linker region of EB1. Moreover, the presence of RNA inhibits EB1 binding to polymerized microtubules in a co-sedimentation assay. RIP-Seq with GFP labelled EB1 from *Drosophila* oocytes showed an enrichment for transcripts involved in cell communication, metabolism, cell cycle, localization and cytoskeletal organization. Further validation of candidate RNAs using single molecule FISH is ongoing.

P1923/B164

Reconstitution and Function of Tubulin Methylation by SETD2.

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Reconstitution and Function of Tubulin Methylation by SETD2 Sarah Kearns[1,2,*], Michael Cianfrocco[2,3], and Kristen Verhey[4] [1.] Chemical Biology Program, University of Michigan, an n Arbor U.S.A. [2.] Life Sciences Institute, University of Michigan, an n Arbor U.S.A. [3.] Department of Biological Chemistry, University of Michigan, an n Arbor U.S.A. [4.] Department of Cellular and Developmental Biology, University of Michigan, an n Arbor U.S.A. * Corresponding author: skearns@umich.edu Microtubules form one of the major components of the eukaryotic cytoskeleton. Assembled from dimers of alpha and beta tubulin, microtubules are involved in a wide variety of cellular functions that range from transporting cargo, forming the mitotic spindle, and creating primary cilia and flagella. How microtubules achieve this functional diversity is partially driven by a “Tubulin Code” of tubulin isotypes and post-translational modifications (PTMs). So far, PTMs including, but not limited to, detyrosination, polyglutamylation, and acetylation are found enriched on specialized microtubule structures and their enzymatic writers and erasers have been identified. Recently, methylation has joined the ranks as a tubulin modification. This mark occurs on spindle and midbody microtubules, and is written by canonically histone methyltransferase SET Domain-containing Protein 2 (SETD2). The role of SETD2 in a histone methylating context has been well studied and linked to cancer phenotypes, but this new finding suggests that tubulin methylation, or lack thereof, drives cancer independently. To investigate SETD2’s tubulin methylation activity and cellular output, we are combining biochemical, kinetic, single-molecule, and structural studies. An active truncated construct of SETD2, which contains the catalytic SET domain to the C-terminal SRI domain, methylates both histone and tubulin. A cancer-driving mutation in the SET domain ablates all SETD2 activity regardless of substrate, but a mutation found in the SRI domain reduces tubulin methylation suggesting a domain-dependent substrate activity. Performing mass spectrometry on both purified recombinant single-isotype tubulin and SETD2-modified recombinant tubulin has identified sites of methylation, indicating that we have reconstituted in *vitro* methylation of tubulin. Additionally, our work suggests that SETD2 methylation may preferentially occur on tubulin dimers instead of polymerized microtubules. This provides a target for structural studies using cryo-electron microscopy. Subsequent structure determination will elucidate how the histone methyltransferase SETD2 is able to recognize and modify tubulin in an SRI-domain dependent way, and how methylation impacts the cytoskeletal and mitotic spindle structure.

Assembly and Disassembly of Primary Cilia

P1924/B166

SCF^{Fbxw5} Facilitates Ciliogenesis by Ubiquitylation of Kif2c/MCAK.

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Members of the kinesin-13 family of proteins are microtubule depolymerases that play important roles in various cellular processes such as spindle formation, chromosome segregation and ciliogenesis. Not surprisingly, these proteins are frequently overexpressed in different cancer types often associated with poor patient prognosis. Despite the importance of their correct abundance, remarkably little is known about how they are maintained and regulated by the ubiquitin proteasome system. Using comprehensive substrate screening on protein microarrays, we identified Kif2c as a target of the multi-subunit ubiquitin E3 ligase SCF^{Fbxw5}. By combining different in *vitro* and in *cellulo* assays, we could establish a strong and direct binding of Kif2c to the substrate receptor Fbxw5. Complete reconstitution of the SCF-dependent ubiquitylation reaction in *vitro* shows that Kif2c is polyubiquitylated by the E3 ligase complex in a highly efficient manner predominantly forming K48 linkages. Accordingly, knockdown of Fbxw5 in RPE-1 cells leads to a general stabilization of Kif2c with a most drastic increase of Kif2c levels at centrosomes after serum starvation. Live-cell imaging revealed that centrosomal levels of Kif2c are already elevated directly after exit of mitosis, suggesting that the regulation is not taking place in G_0 but rather during or before mitosis. In line with a negative role of Kif2c in the generation of primary cilia, knockdown of Fbxw5 leads to a block of ciliogenesis in serum-starved cells, a defect that can be completely reverted by simultaneous knockdown of Kif2c. Thus, we propose that regulation of Kif2c by SCF^{Fbxw5} before mitotic exit is important to reduce its centrosomal levels in order to allow proper formation of primary cilia in G_0.

P1925/B167

Non-canonical Hedgehog Pathway Induces Ciliogenesis Via Promoting the Activation of Autophagy and Ofd1 Centriolar Translocation.

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Primary cilia are microtubule-based structures protruding from the basal body at the surface of most animal cells. Cilia are critical signaling hubs during embryonic development and their absence leads to a variety of severe disorders collectively known as ciliopathies. Canonical Hedgehog (Hh), depends on the presence of primary cilia and involves the binding of Hh ligand to Patched1 (Ptch1), allowing Smoothened (Smo) to accumulate at the primary cilium to activate Gli transcription factor. However, non-canonical Hh pathways do not require primary cilia and in some Smo activates other effectors such as $G\alpha_i$ and liver kinase B1 (LKB1). Despite the importance of cilia, our knowledge of upstream regulators of ciliogenesis is very limited, although autophagy has been implicated. Here we describe a non-canonical Hh pathway as a novel regulator of ciliogenesis. We show that under conditions of low cell density and in serum-rich conditions, Hh activation can unexpectedly activate autophagy via activating LKB1, and induce cilia formation. By depleting various autophagy regulators, we show that Hh-induced ciliogenesis also relies on autophagy, although activation of autophagy is insufficient for ciliogenesis. Using different Smo agonists that enforce different conformations on Smo, we show that in addition to

autophagy, which removes centriolar satellite OFD1, Smo activation of $G\alpha_i$ promotes dynein-mediated translocation of a portion of OFD1 to the centrioles which is also required for Hh-mediated cilia formation. This work reveals that physiological stimuli can promote ciliogenesis, and that autophagic degradation of OFD1 must be accompanied by its translocation and concentration at the centrosome/basal body for ciliogenesis.

P1926/B168

Centriolar Satellites Are Important for Cilium Assembly, Function and Cell Migration.

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Centriolar satellites have recently emerged as key regulators of centrosome/cilium biogenesis, and their mutations are linked to ciliopathies. However, their precise functions and mechanisms of action remain poorly understood. In our recent study, we generated kidney epithelial cells lacking satellites by genetically ablating PCM1 and investigated the cellular and molecular consequences of satellite loss. Satellite-less kidney epithelial cells still formed full-length cilia but at significantly lower levels. Using these cells, we identified the first satellite-specific functions at cilia, specifically in regulating ciliary content, Hedgehog signaling, and epithelial cell organization in three dimensional cultures. However, other satellite-linked functions, namely proliferation, cell cycle progression and centriole duplication, were unaffected in these cells. Quantitative transcriptomic and proteomic profiling revealed that loss of satellites scarcely affects transcription, but significantly alters the proteome. GO-term analysis of biological processes shows that actin cytoskeleton, cell migration and adhesion are affected in satellite-less cells. In the light of this information, we performed wound healing assay and we observed that satellite-less cells are slower to close the wound. Our findings show that satellites have role in efficient cilium assembly and function as well as cell migration.

P1927/B169

Defining the Role of Rab34 GTPase in the Assembly and Function of Primary Cilia.

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Ciliary membrane trafficking is critical to cilium formation and function but remains incompletely understood. Here we describe the role in ciliogenesis of Rab34, a poorly characterized member of the Rab family of GTPases, which have widespread roles in intracellular trafficking. We identified Rab34 in a genome-wide screen for regulators of Hedgehog signaling, and we show here that Rab34 localizes to the Golgi, mother centriole, and cilium and is required for cilium formation. An alysis of Rab34 KO cells indicates that Rab34 is dispensable for some early steps in ciliogenesis but necessary for elaboration of the ciliary membrane and subsequent axoneme extension. By identifying novel mutant alleles of Rab34 that alter nucleotide binding and GTPase activity, we find that ciliogenesis is dependent upon both GTP binding and hydrolysis by Rab34. In particular, GTP-locked mutants of Rab34 localize to the mother centriole and dominantly inhibit cilium formation. Furthermore, when inducibly expressed in ciliated cells, these mutants provoke cilium resorption. Thus, Rab34 is required not only for cilium assembly but also for cilium maintenance. To investigate regulators and effectors of Rab34, we identified Rab34-interacting proteins by affinity purification and mass spectrometry. These purifications have revealed a ciliary trafficking complex as a candidate Rab34 effector and a candidate GTPase activating protein (GAP). Taken together, our findings lead us to propose a model in which Rab34 regulates formation and maintenance of the ciliary membrane through dynamic interaction with ciliary trafficking factors. More

broadly, our studies on Rab34 illustrate the value of genome-wide screening approaches to study cilia and suggest a new gene that may be mutated in ciliopathies.

P1928/B170

Casein Kinase 2 Opposes TTBK2 Function in Regulating Cilium Stability.

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Primary cilia are critical mediators of cellular signaling pathways. The biogenesis of these microtubule-based structures is a multi-step process of which Tau Tubulin Kinase 2 (TTBK2) is a key regulator. TTBK2 is essential for the initiation primary cilia assembly, acting upstream of the intraflagellar transport (IFT) machinery, and embryos homozygous for null mutations to *Ttbk2* fail to assemble cilia. Additionally, embryos with hypomorphic alleles of *Ttbk2* exhibit shorter cilia with perturbed localization of IFT proteins, revealing that TTBK2 is also required to regulate cilia stability. Although TTBK2 is at the nexus of cilia regulation, the molecular pathway in which it functions remains largely undefined. To identify additional proteins that function in the TTBK2-dependent ciliogenesis pathway, we performed screens for both physical and functional TTBK2 interactors. Through these approaches, we identified Casein Kinase 2 Alpha 1 (CSNK2A1) as a negative regulator of TTBK2. We showed that *Csnk2a1* knockout in *Ttbk2* hypomorphic mutant cells rescued defects in cilia formation and length. CSNK2A1 was localizes to the centrosome and enriched at the mother centriole, where it partially co-localizes with TTBK2. We also found that CSNK2A1 physically interacts with TTBK2. CSNK2A1 is required to regulate cilia length and stability: cilia of *Csnk2a1* knockout cells are longer than those of controls, and exhibit instability, particularly at the tip. Live imaging revealed more frequent breakage events in *Csnk2a1*-depleted cells compared to control cells. The composition of these mutant cilia was also perturbed with an abnormal accumulation of IFT proteins, preferentially at the distal part of the cilium. De novo mutations of *Csnk2a1* are linked to a newly characterized disorder, Okur-Chung neurodevelopmental syndrome. Patients diagnosed with this autosomal dominant disorder have dysmorphic facial features and a range of neurodevelopmental abnormalities. We found that stable over-expression of these *Csnk2a1* variants in wild-type cells also led to ciliary tip breakages, similar to those observed in the *Csnk2a1*-depleted cells. From these studies, we identify a new role of CSNK2A1 in cilia stability and trafficking that opposes TTBK2 function. We expect to unveil new mechanisms involved in cilia integrity and trafficking, which will help uncover the impact of cilia instability in human disease and reveal new roles of primary cilia in neural function and homeostasis.

P1929/B171

A Novel TrappII Complex Protein C7orf43/TRAPPC14 Functions in Ciliogenic Vesicle Tethering to the Mother Centriole.

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The Rab11-Rab8 cascade functions in early stages of ciliogenesis. Rab11 traffics the guanine nucleotide exchange factor (GEF) Rabin8 to the centrosome to activate Rab8, needed for ciliary growth. Rabin8 also requires the Transport Particle Protein complex (TRAPPC) proteins for centrosome recruitment during ciliogenesis. Here, we identify C7orf43, detected in Rabin8 mass spectrometry analysis, and show its requirement for ciliation in human cells and zebrafish embryos. C7orf43 directly binds to Rabin8, and its knockdown diminishes Rabin8 ciliogenic centrosome accumulation. Interestingly, C7orf43 co-sediments with TRAPPII complex subunits and directly interacts with TRAPPC proteins. Our findings establish that

C7orf43 is a TRAPPII-specific complex component, referred to as TRAPPC14. Additionally, we show that TRAPPC14 is dispensable for TRAPPII complex integrity, but mediates Rabin8 interaction with the TRAPPII complex. Finally, we demonstrate that TRAPPC14 interacts with distal appendage proteins FBF1 and CEP83, which we show function in GFP-Rabin8 centrosome accumulation, supporting a role for the TRAPPII complex in tethering preciliary vesicles to the mother centriole during ciliogenesis.

P1930/B172

Primary Cilium Assembly Is Initiated by a Rab11 Effector Switch Regulated by Akt Signaling.

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One of the earliest cilia assembly initiating events reported to date is the Rab11-dependent vesicular transport of Rabin8, a Rab8 GEF, to the mother centriole leading to Rab8 activation and cilium growth. This preciliary trafficking of Rabin8 is observed within minutes of serum starvation, which is used to stimulate cilia growth in cultured cells, yet, the serum factors associated are unknown. Here, we report the identification of the serum mitogen lysophosphatidic acid (LPA) as a negative-regulator of ciliogenesis initiation. We demonstrate that LPA, through the LPA receptor 1 (LPAR1), regulates Rab11-Rabin8 interaction and ciliogenesis trafficking via downstream Akt activation, independent of effects on cell proliferation and the cell cycle. Rab11 interaction analysis led to the discovery that Akt directly stabilizes Rab11 binding to its effector WDR44/Rabphilin11, which is shown to negatively regulate Rabin8 preciliary trafficking. Consistent with these observations expression of a WDR44, Akt-site phospho-mimetic, blocks ciliogenesis in human cells and zebrafish embryos. We further characterized Rabin8 preciliary trafficking regulation, demonstrating that FIP3, another Rab11 effector, is required to switch from a Rab11-WDR44 complex to the ciliogenic Rab11-FIP3-Rabin8 complex. Finally, we demonstrate that Akt regulates additional ciliogenic processes downstream of the Rab11 effector switch associated with Rab8-dependent cilia growth. Together, this study uncovers a previously unknown mechanism whereby serum mitogen signaling regulates preciliary trafficking to the mother centriole and ciliogenesis initiation via signaling through the Akt pathway.

P1931/B173

Membrane Tubulation Orchestrates Emergence of the Developing Cilium Via Microtubules Revealed by 3-d Ultrastructure Imaging.

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Cystokeleton and membrane organization is essential for proper assembly of the microtubule-based cilium that occurs, in part, intracellularly in many cells types including photoreceptors and fibroblasts. Yet, the sequence of steps coordinating early ciliogenesis remains poorly understood, and in particular how the developing intracellular cilium membrane fuses with the cell surface membrane for its signaling function is completely unknown. Ciliogenesis requires initial membrane trafficking to the mother centriole (MC) distal appendages followed by docking of vesicles, which will fuse into a larger ciliary vesicle (CV), a pre-requisite step before axonemal growth. Further CV reorganization into the ciliary sheath double membrane is followed by fusion of the latter with the plasma membrane and emergence of the cilium outside the cell. Here, we demonstrate that F-BAR domain containing proteins PACSINs

coordinate membrane reorganization with EHD1 during early ciliogenesis. PACSINs function with EHD1 at the CV step to uncap the mother centriole, and remarkably, stimulate the formation of membrane tubules that contain the ciliary membrane growth regulator Rab8. Correlative light and electron microscopy was performed with Focused Ion Beam Scanning Electron Microscopy (FIB-SEM) to generate 3-D ultrastructure images of the developing cilia and reveal that these membrane tubules connect the CV and ciliary sheaths to the plasma membrane forming an extracellular membrane channel (EMC). Live cell imaging in both human cells and developing zebrafish embryo demonstrated that these tubules originate from the intracellular developing ciliary membranes, and depend on PACSINs and microtubules for their formation. Interestingly, these tubular membranes were also observed forming from the ciliary pocket membrane suggesting a role in trafficking in mature cilia. This work sheds new light on the mechanism of how the developing cilium emerges from inside the cell and exposes the ciliary membrane to the extracellular environment highlighting the importance of the interplay between membrane trafficking and the cytoskeleton during ciliogenesis.

P1932/B174

Single-molecule Tracking Reveals Complex Motility of Transmembrane Proteins in the Chemosensory Cilia Of*C. Elegans*.

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Cilia are vital for the cell's ability to sense its environment and rely on a process called intra flagellar transport (IFT) for their development, maintenance and function in signal-transduction. In IFT, motor proteins transport ciliary components along the polarized microtubule axoneme of the cilium. Among the cargoes of IFT are transmembrane proteins involved in signal transduction. As a model system, we study *C. elegans* chemosensory cilia, which we study using fluorescence microscopy with single-molecule sensitivity. First, we demonstrate that IFT machinery and ciliary components, including TRPV transmembrane channel protein OCR-2 are redistributed away from the ciliary tip upon external chemical stimulation, in a robust, extensive and reversible way. To elucidate the dynamics underlying this dramatic protein redistribution, we performed single-molecule imaging of OCR-2 in live *C. elegans*. Advanced analysis of the single-molecule trajectories shows that, in dendrite and transition zone, active transport is the prevailing motility mode of OCR-2. In the proximal and distal segments, however, motility is a much more complex, location-specific interplay between active transport, normal diffusion and sub diffusion. At the tip, confinement of the membrane proteins plays an important role. Together, our data and analysis demonstrate an intricate interplay between modes of transportation that ensure the proper ciliary distribution of OCR-2. These insights in the dynamics of cellular signal-transduction contributes to a wider understanding of IFT dynamics and to cilia as chemosensory organelles.

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Ciliary/flagellar motility

P1933/B175

Characterization of a Novel Ciliary Protein, CFAP70.

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Cilia- and flagella-associated protein 70 (CFAP70) is a 150 kDa protein discovered by our transcriptome analysis in mice. Little is known about this protein, except for the tetratricopeptide repeat (TPR)

domains concentrated on the C-terminus, which is known to mediate protein-protein interactions, and present in many components of the intraflagellar transport (IFT) complex. We characterized the mouse CFAP70 and *Chlamydomonas* homolog FAP70 with particular interests on the ciliary localization and function. RT-PCR of various mouse tissues demonstrated the association of CFAP70 with motile cilia and flagella, and the immunohistochemistry showed a staining pattern being different from the punctate signals of IFT subunits. A stepwise extraction of proteins from porcine tracheal cilia showed that CFAP70 bound tightly to the ciliary axoneme. Fluorescence microscopy of the cultured ependyma expressing fragments of CFAP70 demonstrated that the N-terminus rather than the C-terminus with the TPR domains was more important for the ciliary localization. When CFAP70 was knocked down in cultured mouse ependyma, reductions in cilia beating frequency were observed. Consistent with these observations, a *Chlamydomonas* mutant lacking the FAP70 showed defects in outer dynein arm (ODA) activity and a reduction in flagellar motility. Cryo-electron tomography revealed that the N-terminus of FAP70 resided stably at the base of the ODA. However, there are no obvious changes in the *FAP70*-null mutant axoneme. These results collectively demonstrate that CFAP70 is a novel regulatory component of the ODA in motile cilia and flagella. Furthermore, binding of CFAP70 to the axoneme takes places at the N-terminus being independent of TPR domain.

P1934/B176

Evolution of Multicellularity - Lessons from Ciliary Waves of a Giant Single-celled Cilate *Stentor Coeruleus*.

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Early multicellular organisms have long been suggested to have evolved from unicellular organisms which can form colonies to achieve mutual benefit. While many unicellular protists (e.g. ciliates) are known to rapidly switch between a solitary unicellular state and colonial multicellular-like states, the rules governing these transitions and the benefits they endow the organisms are still poorly understood. We have developed *Stentor coeruleus*, a giant (~1 mm) unicellular ciliate, as a model organism to investigate unicellular to multicellular transition. *S. coeruleus* beat their oral cilia in a metachronal wave to swim as well as to generate microcurrents that transport dissolved nutrients and smaller prey cells critical for nutrition. These ciliary flows are characterized by symmetric vortices on either side of the oral opening. Further, these organisms can spontaneously self-assemble into hemispherical colonies with oral openings radially radiating outwards. Using a combination of high-speed imaging, particle image velocimetry and computational fluid dynamics, we show that individuals in a colony cooperate to generate faster feeding current as compared to their solitary counterparts. The combined flows can act over longer distances and transport greater amounts of fluid per individual organisms, and thus giving a direct survival benefit to individuals in a colony vs solitary ones. The advantages endowed by their colonial organization can thus potentially provide fundamental insights into the selective forces favoring early evolution of multicellular organization.

P1935/B177

Identifying and Mapping the Protein Composition of the Central Pair Apparatus through Proteomics.

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The central pair (CP) apparatus is a robust yet elusive component of the axonemal structure in motile cilia and flagella. The presence of the CP distinguishes the motile cilia from its non-motile counterpart, the primary cilia. The CP plays fundamental roles in conferring structural integrity, flexibility and regulation of ciliary beating. It consists of two bridged microtubules termed C1 and C2. Both C1 and C2 are decorated with multiple protein complexes that are unique and specific to each microtubule. The protein composition and organization of the CP have yet to be addressed in full. This is in part due to previous approaches which relied on existing Chlamydomonas mutants to identify CP proteins one by one. In this study, we looked to identify new CP proteins and localize all the new and currently known CP proteins in a more comprehensive fashion using a proteomic approach. The proteomic composition of the entire CP was identified by comparing mass spectrometry (MS) of wild type cells and pf15 cells, which is missing the entire CP structure. To map CP proteins into sub-structures, Chlamydomonas reinhardtii strains of different CP components, were subjected to MS analysis. Mutants missing parts of CP structure: pf16 (C1-less strain), pf6 (missing C1a complex) and cpc1 (lacking C1b complex), were used to generate MS profile of each CP protein and, consequently, for mapping into sub-structures based on known proteins. Flagella were purified from wild type and mutants; pf15, pf16, pf6 and cpc1, treated with 0.6M NaCl twice to remove dynein motor complexes, and analyzed by MS. MS was performed by in-gel digestion 3 times for each strain and statistical analysis was performed. Comparison between wild type and pf15 MS results revealed over 40 new CP protein candidates in addition to well-characterized CP proteins. By comparing these results to subsequent mutant MS results, those proteins were further mapped to the sub-structures surrounding the CP. We believe that our approach has provided further insights into the composition and localization of new proteins of the CP.

P1936/B178

Multi-scale Spatial Heterogeneity in the Airway Multiciliated Epithelium Underlies Directed Flow Generation.

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Flow generation is essential for the development and physiology of organisms across the tree of life. Flows required for processes that range from swimming in single cell organisms to mucus clearance in humans are generated by arrays of motile cilia. To generate large scale fluid flows, motile cilia must coordinate their activity across entire tissues. The mechanisms of fluid transport have been studied extensively at the level of the individual cilium and collectively moving metachronal waves. However, the connection between local cilia arrangement and the patterns of flow they generate remains largely unexplored. Here, we image the mouse airway multiciliated epithelium from the sub-cellular (nm) to the organ scales (mm), characterizing quantitatively its ciliary arrangement and the flow generated. Locally we measure heterogeneity in both cilia organization and flow structure, but we find that fluid transport is coherent and globally directed across the trachea. To examine this result, we developed a hydrodynamic model and explore systematically the flows generated by diverse ciliary architectures.

Surprisingly, we find that disorder enhances particle clearance, whether it originates from fluctuations, heterogeneity in multiciliated cell arrangement or ciliary misalignment. Thus, disorder in ciliary arrays provides a mechanism to generate globally directed flows that are highly correlated in space and time. Altogether our results shed light into how multi-scale patterning of an array of multi ciliated cells determines its emergent dynamics. Furthermore, this work is also applicable to understand the origin of human airway pathologies, which are the third leading cause of deaths worldwide.

P1937/B179

Ccdc113 / Ccdc96 Complex Links N-drc, Rs3 and Idas G Tailand Is Essentialfor Proper Cilia Beating.

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A proper ciliary beating requires the coordinated activity of numerous axonemal complexes. While the protein composition and a role of the major axonemal complexes is well established, little is known about the minor complexes and linkers, which connect the major complexes and likely regulate or coordinate their activity and/or mediate signal transduction. We identified a structure that connects the N-DRC, the tail of the IDA g, and the base of the RS3, and showed that it is composed of the evolutionarily conserved proteins, Ccdc113 and Ccdc96. The ciliary localization of Ccdc96 and Ccdc113 is interdependent and loss of Ccdc113 or Ccdc96 alters cilia beating. We propose that the Ccdc113/Ccdc96 complex transmits signals from N-DRC and RS3 to IDA g and thus, regulates its activity and the cilium beating pattern. Additionally, we observe that the density of the Ccdc113/Ccdc96 complex contributes to an elongated linker structure that, passing through the N-DRC, the MIA complex, and the IC-LC complex of IDA f connects all major components of the 96-nm repeat.

P1938/B180

Changes in the Helical Symmetry of the Axoneme of *Chlamydomonas* Flagella Coupled with Ca^{2+} Concentrations.

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X-ray diffraction allows the extraction of structural information from unfixed biological materials that remain functional in an aqueous environment and allows dynamic or time-resolved measurements on the structures of biological materials. It is particularly useful when the target molecules to be investigated are periodically arranged because X-rays scattered by periodic arrays interfere with each other to generate strong signals. An axoneme with its highly ordered structures is, therefore, a suitable target for X-ray diffraction. Using *Chlamydomonas* as the useful model system for the structural analysis of eukaryotic cilia and flagella, we have explored the spatial arrangement of axonemal components and helical symmetry of the axoneme under physiological conditions by small-angle X-ray fiber diffraction. The axonemes were oriented in a physiological solution by continuous shear-flow and were exposed to intense and stable X-rays generated in the synchrotron radiation facility SPring-8 BL40XU. When the diffraction patterns were viewed along the meridian, the peaks of the meridional reflections were sharp and well-separated from each other and could be indexed to a basic axial repeat of 96 nm, which was the periodicity of the radial spokes and inner dynein arms. The reflections were observed up to the 12th (at 1/8 nm^{-1}) and 24th (at 1/4 nm^{-1}) orders. The use of a variety of mutants lacking specific axonemal

components allows us to assign the reflections to the structures since diffraction patterns from mutant axonemes exhibited a systematic loss/attenuation of meridional/layer line reflections. Next, we explored the spatial arrangement and dynamics of axonemal components under various [Ca^{2+}]. At the low [Ca^{2+}], 24-nm meridional reflections split each into the layer lines, suggesting changes in the helical nature of nine doublet microtubules in the axoneme. At the high [Ca^{2+}], the intensity of layer lines decreased. This change found in wt axonemes was also observed in the central pair-less mutant, *pf18*. Furthermore, the dependency of the change in diffraction patterns upon [Ca^{2+}] was similar to that of the waveform changes observed in *Chlamydomonas*. These results suggest the possibility that [Ca^{2+}] changes the helical arrangement of doublet microtubules in an axoneme and modulates the coordinates of dynein arms and the adjacent doublets. This work was supported by Grant-in-Aids for Scientific Research (C), the Japan Society for the Promotion of Science (JSPS, grant numbers 26440089 and 17K07376 to K.O.), and the Takeda Science Foundation and Hyogo Science and Technology Association (K.O.).

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Centrosome Assembly and Functions 2

P1939/B182

A Perinuclear Microtubule-Organizing Center Controls Plasma Membrane Growth and Basement Membrane Secretion by Engaging a Novel Paradigm for Microtubule Assembly.

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Non-centrosomal microtubule-organizing centers (ncMTOCs) are a prevalent feature of differentiated cell types. ncMTOCs are diverse in their sites of subcellular assembly, yet little is known of their structures, regulation, and what functions they serve in the specialized cell types where they are found. Here we show a unique molecular architecture for the ncMTOC in fat body cells, a Drosophila differentiated cell type that has critical secretory functions that we show are controlled by the ncMTOC. We show that a prominent ncMTOC is assembled at the nuclear surface in the fat body, which is the functional equivalent of vertebrate liver and adipose tissue. This ncMTOC has several novel features and mechanisms of MT assembly that distinguish it from centrosomes and other MTOCs. Firstly, this MTOC is remarkable in its complete lack of dependence on gamma-tubulin, the broadly employed MT nucleator at centrosomes and ncMTOCs. Secondly, we show that the Nesprin Msp300 establishes the MTOC on the nuclear surface together with the spectraplakin Shot. This complex recruits the MT minus-end regulators Patronin (CAMSAP homolog) and Ninein, which function redundantly to establish MT nucleation at the nuclear surface. Thirdly, Patronin + Ninein recruit the MT polymerase Msps (XMAP215 homolog) to generate the radial MTs upon which membrane trafficking occurs. We further show by co-IP that Patronin and Msps reside together in a complex. Overall, this MT regulatory axis involving Patronin + Ninein to stabilize or anchor MT minus ends while recruiting the MT polymerase Msps to assemble the radial MT array, anchored at the nucleus by Msp300 + Shot, and functioning independently from gamma-tubulin, represents a novel paradigm for MTOC structure and function. Surprisingly little is known about the cell biological or physiological functions that diverse ncMTOCs serve in the variety of cell types where they are found. Here we show that the fat body perinuclear ncMTOC controls membrane trafficking, impacting plasma membrane homeostasis. The microtubule arrays radiating from the perinuclear ncMTOC coordinate dynein motor-dependent endocytic trafficking

to maintain proper plasma membrane growth, representing the first example of microtubule-mediated control of plasma membrane growth as far as we are aware. Importantly, the uncontrolled plasma membrane overgrowth that results from disruption of the ncMTOC causes extracellular entrapment of large secreted complexes like collagen within the convoluted membrane folds, a phenomenon comparable to liver fibrosis.

P1940/B183

CK2-dependent Phosphorylation Regulates ZYG-1/PLK4 Stability and Centrosome Number.

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Spindle bipolarity is critical for genomic integrity and centrosome number often determines bipolarity in mitosis. Tight control of centrosome assembly is, therefore, vital for the fidelity of cell division. The kinase ZYG-1/Plk4 is a key centrosome regulator and ZYG-1/Plk4 protein levels appear to be regulated through opposing activities between kinases and phosphatases in *C. elegans* embryos. Our prior work showed that Casein Kinase II (CK2)-dependent phosphorylation influences centrosome-associated ZYG-1 levels and CK2 acts as a negative regulator of centrosome assembly. We thus postulated that CK2 directly phosphorylate ZYG-1 and CK2-dependent phosphorylation influences ZYG-1 stability and centrosome-associated ZYG-1. To test our hypothesis, we used in *silico* tools and identified serine 279 (S279) and several adjacent serine residues within ZYG-1 as putative CK2 target sites. S279 is located within a linker domain (L1) between the kinase domain and cryptic polo box in ZYG-1. The L1 domain is known to be critical for ZYG-1 loading to centrosomes and its interaction with another centrosome factor SAS-6. Using CRISPR/Cas9 genome editing, we mutated these serine residues to alanine (A: non-phosphorylatable) or aspartic acid (D: phospho-mimetic), and tested for the effects of site-specific phosphorylation of ZYG-1 in the early *C. elegans* embryo. Our data show that non-phosphorylatable ZYG-1 mutations restore centrosome duplication and embryonic viability to hypomorphic *zyg-1* mutants, whereas phospho-mimetic mutations aggravated *zyg-1* phenotypes. We also observe that phospho-mutants of ZYG-1 at multiple residues (4A and 4D) exhibit much stronger effects than single-site mutation at S279. Consistent with our hypothesis, non-phosphorylatable mutations result in elevated ZYG-1 levels and supernumerary centrosomes in early *C. elegans* embryos. In contrast, phospho-mimetic ZYG-1 mutations lead to the reduced centrosomal ZYG-1 levels and often to a centrosome duplication failure. We also show that blocking the 26S proteasomal activity leads to a partial rescue of centrosome duplication to the phospho-mimetic ZYG-1 mutant, consistent with our hypothesis that phosphorylated ZYG-1 is subject to proteasomal destruction. Finally, our in *vitro kinase* assay indicates that CK2 may directly phosphorylate ZYG-1 at multiple sites. Together, our results suggest that CK2-dependent ZYG-1 phosphorylation at multiple sites contributes to ZYG-1 stability via the 26S proteasomal degradation. Thus, ZYG-1 phosphorylation by CK2 provides an additional mechanism to maintain ZYG-1 activity, leading to one and only one centrosome duplication during the early *C. elegans* cell division.

P1941/B184

Identification and Characterization of Sas-6 as an Mps1 Substrate for Centriole Biogenesis.

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The faithful segregation of chromosomes during mitosis is essential for maintaining genetic stability. Improper segregation leads to aneuploidy and contributes to genetic instability and tumorigenesis, common features in cancer. The centrosome is an organelle composed of a pair of centrioles made up of triplet microtubules organized in a cylindrical structure that has nine-fold symmetry. Centrosomes function as the poles of the mitotic spindle to ensure equal segregation of chromosomes and safeguard against genetic instability. Like DNA, centrosomes are duplicated once and only once per cell cycle, wherein the two centrioles of a single centrosome each serve as the template to nucleate their own daughter centrioles in the centriole assembly pathway. Once the daughter centrioles are assembled, the two mother/daughter pairs separate from each other to form two centrosomes that go on to organize the mitotic spindle. Failure to regulate this process can lead to the amplification of centrosomes and increases the likelihood of aberrant spindle formation in mitosis and the improper segregation of chromosomes. The protein kinase Mps1 is necessary for the spindle assembly checkpoint and contributes to centriole assembly in animals. We have previously shown that preventing the degradation of Mps1 at centrosomes led to the overproduction of centrioles, though the exact mechanism remains to be seen. Mps1 phosphorylates the centrosomal protein Centrin2 and targets it to the centrosome, but whether Centrin2 is the only centrosomal substrate of Mps1 is not known. To address this question, we have looked at several centrosomal proteins involved in the centriole assembly pathway for interactions with Mps1. We have found that Sas-6, a protein involved in the formation of the cartwheel base of the centriole and the source of its nine-fold symmetry, is a substrate of Mps1. By mass spec, we have identified 5 in vitro Mps1 phosphorylation sites in Sas-6. Single site mutations to non-phosphorylable or phospho-mimetic residues does not affect the localization of Sas-6 or its ability to recruit the microtubule nucleating protein gamma-tubulin. While mutating all five sites similarly does not affect Sas-6 localization or function, we found that the Sas-6 5D phospho-mimetic mutant display defects in the overproduction of centrioles during an extended S-phase arrest. These results support the identification of Sas-6 as a novel and relevant substrate of Mps1 and will require further research to unravel their interactions and effects on the regulation of centrosome duplication.

P1942/B185

Genetic an alysis of a SAS-6 Mutant Suggests an Instructive Role for the Mother Centriole in Centriole Assembly.

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Centrioles are nine-fold symmetric barrel-shaped organelles that exist as mother-daughter pairs and participate in the assembly of bipolar spindles as well as in the formation of cilia and flagella. In canonical duplication, centrioles are precisely duplicated through a process that involves the formation of a new daughter next to each preexisting mother centriole. It is well established that mother centrioles provide spatial control by recruiting initiation factors, but current evidence is inconclusive about whether the mother provides any additional assistance. Arguments against this are supported by the finding that centrioles are able to form in the absence of a mother centriole, called de novo. Additionally, SAS-6, the protein responsible for forming the centriole scaffold, has been shown to

possess the ability to self-oligomerize with nine-fold radial symmetry in vitro, providing a mechanism for which daughter centrioles could self-assemble. However, de novo centrioles are often structurally defective and lack numerical control, suggesting that the mother centriole may play a more instructive role in centriole assembly. Here we describe a missense (D9V) mutation in *C. elegans* SAS-6 that specifically compromises the ability of centrioles to act as mothers. The SAS-6(D9V) protein can be assembled into structurally-defective centrioles (D9V centrioles) that organize normal-sized centrosomes and spindle poles. Importantly, D9V centrioles are fully capable of initiating daughter centriole formation, as evidenced by their ability to recruit near-normal levels of an essential assembly initiation protein, SAS-7, as well as by their ability to reliably form daughters when exposed to a cytoplasmic pool of wild-type SAS-6 protein. However, in the presence of a cytoplasmic pool of SAS-6(D9V) protein, D9V mother centrioles frequently fail to form daughters. In contrast, under these same conditions, wild-type mothers efficiently form daughters, demonstrating that SAS-6(D9V) protein is assembly competent. Together these data indicate that centriole duplication failure associated with SAS-6(D9V) arises in part from a defect in a post-inititation role of the mother centriole, and suggest some type of molecular communication between the mother centriole and the assembling daughter.

P1943/B186

Regulation of Centriole De Novo Biogenesis.

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In proliferating cells, centrosome formation via canonical duplication is spatially, temporally and numerically regulated by the presence of mature centrioles. However, in several eukaryotic cell-types, centrioles assemble de novo*,* yet very little is known regarding the regulation of this process, in part because previous studies lacked the appropriate techniques to look into this process in live samples. Overexpression of Polo-like kinase 4 (Plk4) triggers de novo assembly of multiple centrioles in the cytosol of unfertilised *Drosophila melanogaster* eggs. We have established an ex vivo assay for high-resolution confocal live-imaging of centriole assembly within small cytosolic explants from unfertilised fly eggs, allowing us to investigate how this process is spatially and temporally regulated. Surprisingly, we found that both canonical duplication and de novo pathways co-occur within the same cytoplasmic explant, at their own temporal kinetics suggesting that either process does not inhibit the other. We followed centriole de novo biogenesis in time and determined where and when centrioles formed in the droplets. Comparing our observations to stochastic models demonstrated that recently formed centrioles do not impact the location where new centrioles assemble de novo, at high Plk4 levels. We observed that after an initial temporal delay, centrioles assemble at a high rate (0.15-0.75 min^{-1}) that accelerates over time. Our experimental results indicate that this burst in biogenesis is not due to a cell-cycle dependent mechanism but, instead, Plk4 concentration - and presumably its activation - are the main driving force regulating the process. In agreement, altering Plk4 concentration delays the onset of centriole biogenesis. Altogether, our results show that Plk4 local concentration is critical in controlling the onset of centriole de novo formation and its temporal kinetics.

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A Novel Assay to Screen Sirna Libraries Identifies Protein Kinases as Required for Chromosome Transmission.

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One of the hallmarks of cancer is chromosome instability (CIN), which leads to aneuploidy, translocations and other chromosome aberrations. However, in the vast majority of human tumors the molecular basis of CIN remains unknown, partially because not all genes controlling chromosome transmission have yet been identified. To address this question, we have developed an experimental high-throughput imaging (HTI) siRNA assay that allows the identification of novel *CIN* genes. Our method uses a human artificial chromosome (HAC) expressing the *GFP* transgene. When this assay was applied to screen a siRNA library of protein kinases we identified *PINK1*, *TRIO*, *IRAK1*, *PNCK,* and *TAOK1* as potential novel genes whose knockdown induces various mitotic abnormalities and results in chromosome loss. The HAC-based assay can be applied for screening different siRNA libraries (cell cycle regulation, DNA damage response, epigenetics, transcription factors) to identify additional genes involved in CIN. Identification of the complete spectrum of *CIN* genes will reveal new insights into mechanisms of chromosome segregation and may expedite the development of novel therapeutic strategies to target the *CIN* phenotype in cancer cells.

P1945/B188

Evidence That Centrioles Duplicate Via an Inheritable Organelle Zone.

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During the cell cycle, organelles must undergo biogenesis to increase in amount and be inherited into daughter cells. Although the molecular mechanisms that regulate organelle biogenesis is under active investigation, current knowledge of how their inheritance works is limited. Our recent studies in *Drosophila* embryos have revealed essential aspects of how centriole biogenesis is regulated as a function of time (Aydogan et al. 2018 and 2019). However, the spatial control over how a mother centriole initiates the growth of its daughter, and whether this information is inherited to the next generation, remains unclear. Here we present our recent evidence that centrioles may acquire a permanent, inheritable metabolite pool that is required for the sustained duplications of centrioles in flies. Live-imaging experiments using spinning disk confocal microscopy showed that cartwheel assembly proteins Sas-6 and an a2/STIL are present in excess on the mother centriole prior to the formation of the daughter. Further investigation with Airyscan super-resolution microscopy demonstrated that the excess Sas-6 and an a2 form a metabolite pool that is associated with, but separate than, the overall structure of mother centrioles. Our results suggest that this metabolite pool is permanently maintained on the mother centriole throughout the cell cycle, but is replenished and inherited to the daughter centriole presumably when the daughter separates from its mother to become a new mother itself. We reasoned that this metabolite pool might function to serve as buffer that limits the parameters of

daughter centriole growth (such as rate or period), and/or to help mark a permanent, inheritable site on the mother centriole that allows efficient duplication of the daughter centriole. In order to investigate this hypothesis, we first looked at whether the size of this metabolite pool is correlated with parameters of centriole growth, and found that this was not the case. To more directly test this, we developed a novel computational script that allows looking at the same parameters distinctly on old and new mother centrioles, but we again observed no significant difference between the two. In agreement with these findings, we found that old and new mothers recruit similar amounts of Plk4, an essential kinase that homeostatically controls the rate and period of centriole growth. Furthermore, unlike Sas-6 and an a2, we found that Plk4 is absent from the metabolite pool, potentially suggesting that this pool does not necessarily serve as buffer to limit aspects of daughter centriole growth. These indicate that the metabolite pool associated with centrioles in cycling cells may form a permanent, inheritable organelle zone on the mother centriole that allows efficient duplication of the daughter centriole.

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A Molecular Mechanism for Assembly of the Pre-procentriole.

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Centriole duplication begins with the accumulation of several assembly factors at a single asymmetric spot on mother centrioles, which eventually gives rise to a procentriole. Specifically, this involves the hierarchical recruitment of a conserved set of centriole proteins, including Polo-like kinase 4 (Plk4)/ZYG-1 and an a2/SAS-5/STIL, followed by the cartwheel protein Sas6. Recruitment of these proteins occurs during late mitosis in *Drosophila* cells, first forming a nebulous structure known as the 'pre-procentriole' and later, during the subsequent S-phase, assembles into a procentriole containing a central cartwheel and a cylinder of microtubule bundles. Because the pre-procentriole is the progenerative platform for nascent centriole growth, our study focuses on determining the molecular details of its composition, structure and assembly, which are largely unknown. Initially, Plk4 decorates the surface of mitotic centrioles due to its interaction with a centriole-targeting factor. Next, an a2/STIL is recruited and stimulates Plk4 kinase activity. In turn, an a2/STIL's C-terminal STAN domain is phosphorylated by Plk4, generating a high-affinity phospho-binding site for Sas6 loading. Although the assembly steps immediately preceding Sas6-loading appear clear, the mechanism underlying the upstream pre-procentriole recruitment of an a2/STIL is not. In contrast to proposed models of an a2/STIL recruitment, we recently showed that loading of *Drosophila an* a2 onto procentrioles is actually independent of Plk4 binding, but does require Plk4's catalytic activity. We have discovered that the amyloid-like domain of Sas4, a centriole surface protein, binds Plk4 and an a2. Sas4 binding facilitates phosphorylation of an a2's N-terminus which increases an a2's affinity for Sas4. Consequently, an a2 accumulates at the procentriole to induce daughter centriole assembly. We propose a new multi-step pathway for the initial assembly of the pre-procentriole and provide insight into how Sas4 may define the site of daughter centriole assembly.

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Inner Nuclear Membrane Proteins Lem2 and Sad1 Regulate Localized Nuclear Envelope Remodeling during Mitosis to Insert the Fission Yeast Centrosome.

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Making sure the centrosomes in a cell have access to the chromosomes to ensure the correct division of nuclear material is vital during mitosis. In fission yeast, the centrosome (called the spindle pole body, SPB) is assembled in the cytoplasm adjacent to the nuclear envelope (NE). To access the chromosomes during mitosis, fission yeast breaks down the NE. However, this NE breakdown is highly localized, restricted to underneath the SPB and allows insertion of the SPB into the NE. How this process of localized NE disassembly occurs is not well understood, particularly since fission yeast lack many components of the lamin network thought to trigger NE breakdown in metazoans. Here, we utilized structured illumination microscopy (SIM) to visualize the organization of SPB and NE proteins during mitosis in *Schizosaccharomyces pombe* to understand how localized and highly regulated NE breakdown occurs. The high spatial resolution of SIM allows us to delineate between proteins that are localized to the SPB core and proteins that localize to a ring around the SPB. Further analysis of protein localization using single particle averaging methods allowed us to determine if NE proteins are enriched in nuclear regions near the SPB, suggesting a function in NE breakdown and/or remodeling. We further examined the function and hierarchical recruitment of proteins to the SPB region to better understand their role in localized NE breakdown and SPB insertion. Our data supports the idea that Lem2 and Sad1 play important, non-redundant roles in the control of membrane organization at the SPB, acting as upstream regulators of membrane remodeling.

P1948/B191

Cep57 Interacts with Cep63 to Construct the Inner Layer of Pericentriolar Matrix and Provide a Platform for Plk4-dependent Centriole Biogenesis.

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Cep57, initially identified as an intracellular trafficking mediator of FGF-2, has been characterized as a part of a pericentriolar complex containing Cep63 and Cep152. A recent study demonstrated that Cep63 and Cep152 form a heterotetrameric complex that self-assembles into a cylindrical higher-order assembly around a centriole. This assembly process is critical for subsequent recruitment of Polo-like kinase 4 (Plk4), a key regulator for centriole duplication, to the future procentriole assembly site. How Cep57 interacts with the Cep63-Cep152 complex and contributes to the structure and function of the Cep63-Cep152 self-assembly remains unknown. Here we showed that depletion of Cep57 delocalizes Cep63 from centrosomes and cripples Sas6 recruitment to the procentriole assembly site. An alyses with cells derived from Cep57-knockout mouse embryonic fibroblasts confirmed this finding. Subsequent coimmunoprecipitation analyses with transfected lysates revealed that the N-terminal region of Cep57 interacts with the N-terminus of Cep63. Consistently, cells expressing a Cep57 mutant defective in Cep63 binding failed to properly recruit Cep63 and Sas6 to centrosomes. In disagreement with the previous report, PCNT did not significantly interact with Cep57 under the conditions where Cep63 interacted with it efficiently. Furthermore, Cep57 failed to directly interact with Cep152 but rather associated with the latter via Cep63, suggesting that Cep63 mediates the formation of a trimeric Cep57-

Cep63-Cep152 complex. Immunostaining analyses showed that Cep57 localizes to the inner face of pericentriolar Cep63 signals but at the outskirts of centriolar tubulin signals. Intriguingly, the C-terminal domain of Cep57 has been shown to possess a capacity to directly bind to microtubules. Taken together, we propose that the Cep57-Cep63 interaction is critical to link the Cep63-Cep152 assemblies to the outskirts of Cep57-associated centrioles. We further speculate that this multilayered organization is important to generate a higher-order architectural platform that serves to orderly arrange various pericentriolar components in a confined subcentrosomal space and intricately regulate Plk4-dependent centriole biogenesis.

P1949/B192

Regulation of Plk4- Dependent Centriole Duplication by the Cullin4/DDB1/VprBP E3 Ubiquitin Ligase Complex.

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Centrioles play important functions for the assembly of both centrosomes and cilia. Centriole formation is triggered by and dependent on polo-like kinase 4 (Plk4). Overduplication of centrioles is prevented by degradation of interphase Plk4 mediated by the SCF SLIMB/β-TrCP E3 ubiquitin ligase complex which recognizes a phosphodegron after Plk4 autophosphorylation of multiple residues within a 24- amino acid region. However, a β-TrCP binding site mutant of Plk4 is only modestly stabilized implying the existence of additional E3 ubiquitin ligases that regulate Plk4 levels. We find that the Cullin(CUL)4A/B-DDB1/VprBP E3 ligase complex targets Plk4 for degradation. The DDB1/VprBP complex binds to and ubiquitylates Plk4. Depletion of each VprBP, CUL4A/B or DDB1 causes centrosome amplification, leading to the formation of multipolar mitotic spindles. Furthermore, we show that the interaction between Plk4 and CUL4-DDB1 is independent of Plk4 kinase activity and that VprBP and DDB1 interact specifically with polo-boxes 1 and 2 of Plk4, suggesting that DDB1 promotes Plk4 ubiquitylation independently of the β-TrCP recognition motif. Thus, the SCF SLIMB/β-TrCP pathway which targets Plk4 for ubiquitylation on the basis of its phosphorylation state and the CUL4A/B-DDB1-VprBP ligase which selects Plk4 for ubiquitylation by binding to the conserved PB1-PB2 domain appear to be complementary ways to control Plk4 abundance to prevent centriole overduplication.

P1950/B193

The Relationship between Rab11-endosomes and the Centrosome during Abscission.

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During interphase, Rab11-GTPase-containing endosomes recycle endocytic cargo. However, little is known about Rab11 endosomes in mitosis. Previous work has identified that Rab11 endosomes are utilized during the final stage of cell division, termed abscission, where they are directed into the cytokinetic bridge and dock adjacent to a proteinaceous structure called the cytokinetic midbody. My laboratory has identified that Rab11-endosomes interact with a specific sub-structure of the centrosome, mother centriole appendages. Mother centriole appendages functionally define the “older centriole” of the centriole pair. Our studies herein identify the relationship between Rab11-endosomes and the centrosome throughout the cell cycle. Strikingly, upon anaphase exit, the oldest spindle pole (containing the oldest centriole) and youngest mitotic spindle poles both maintain a population of Rab11-endosomes, but the oldest pole maintains a significantly larger population of endosomes. Using Fluorescent Recovery After Photobleaching (FRAP) we find that bleaching the Rab11-binding protein,

FIP3, at the oldest spindle pole presents with a significantly different recovery rate when compared to the youngest. These studies suggest that spindle pole age may be modulating Rab11-endosome function during cytokinesis. After the cytokinetic furrow has ingressed, formation of a second endosome compartment in each daughter cell occurs proximal to the cytokinetic bridge where the spindle poles remain at the distal ends. The oldest spindle pole then moves proximal to the bridge carrying with it its population of endosomes, where it meets the second endosome compartment. When this pole is adjacent to the bridge, then the youngest pole moves towards the bridge to meet its second endosome compartment. Once both spindle poles are proximal to the bridge, abscission occurs. Rab11-null cells result in transferrin receptor positive endosomes that are unable to track with spindle poles during abscission and result in abscission failure. We present a model where the centrosome may direct Rab11-endosomes into the cytokinetic bridge to initiate abscission.

P1951/B194

Phase Separation Induced by Plk4 Autoactivation and Noncatalytic Domain Clustering Drives Centriole Biogenesis.

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As the main microtubule (MT)-organizing center in animal cells, the centrosome plays a pivotal role in various cellular processes, such as spindle formation, chromosome segregation, and cell division. Tight control of centriole duplication is critical for normal chromosome segregation and the maintenance of genomic stability. Polo-like kinase 4 (Plk4) is well characterized as a key regulator of centriole biogenesis. However, how Plk4 dynamically promotes its symmetry-breaking relocalization and achieves its procentriole-assembly state remains unknown. Here we demonstrated that Plk4 promotes its own ring-to-dot localization conversion by autophosphorylating and transmuting the physicochemical properties of its noncatalytic cryptic polo-box (CPB), thereby causing it to rapidly coalesce into a nanoscale spherical condensate with a distinct constituent phase. An alyses of the crystal structure of a phospho-mimicking, condensation-proficient CPB mutant revealed a disordered loop at the CPB PB2 tip predicted to become an intrinsically disordered region (IDR). Mutations in the putative IDR eliminated phospho-CPB-dependent Plk4 condensation, Plk4's symmetry-breaking ring-to-dot relocalization, and its ensuing centriole biogenesis. In a related experiment, CPB phosphorylation also promoted Plk4's dissociation from the Cep152 tether while binding to downstream STIL, thus allowing Plk4 condensate to serve as an assembling body for centriole biogenesis. Thus, we propose that Plk4 is an unparalleled kinase that harnesses its KD-dependent autophosphorylation onto its CPB to induce phospho-CPB-dependent physicochemical condensation. This unique capacity enables Plk4 to phase-separate into a matrix-like body that can amass downstream components critical for procentriole assembly.

P1952/B195

Dynamic Remodeling of Microtubule-Organizing Centers and Their Requirements for Zika Virus Proliferation.

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The 2015-2016 outbreaks of the Zika virus (ZIKV) in the Americas resulted in a public health crisis due to its links to microcephaly and additional birth defects collectively known as congenital Zika syndrome. ZIKV is a member of the single-stranded RNA Flavivirus family, which includes Dengue and West Nile viruses, that are mainly transmitted to humans through mosquitos. Inherited forms of microcephaly are due to recessive mutations mostly in centrosome protein-encoding genes. However, the functional link between centrosomes and the development of microcephaly remains unclear. The centrosome and the Golgi Apparatus are involved in the formation and anchoring of microtubules in human cells, and they function as microtubule organizing centers (MTOCs). Due to the connection to the centrosome in the inherited forms of microcephaly, we investigated how the cell's MTOCs and their MTs are used by ZIKV to facilitate viral infection and replication. During the Flavivirus infection cycle, the ER rearranges to form a compact structure called a viroplasm that is responsible for virus replication and assembly, and MTs contribute to its formation. We show that the ZIKV viroplasm is toroidal shaped, and MTs form a cage-like structure around the viroplasm and in a dense bundle located at its hollow inner core. We also found a close association between the centrosome and the Golgi MTOC with the viroplasm. The Golgi surrounds the outside of the viroplasm. In addition, the centrosome and a Golgi cluster is located at the hollow core of the ZIKV viroplasm, and the combined MTOCs' activities are elevated at this core. As both MTOCs localize to the area of ZIKV replication in infected cells, we tested the requirement of the centrosome and the Golgi MTOC for viroplasm formation during infection. Viroplasm formation still occurs in cells without the centrosome and without the Golgi MTOC; however, efficiency of infection and viral release may be affected. Though we observe a viroplasm in cells without centrosomes, the formation of its inner core is disrupted. In addition to the host MTOCs' involvement, we investigated which ZIKV factors contribute towards viroplasm organization. Viral proteins play a role in the subcellular rearrangements that occur during infection, and we investigated if individual ZIKV proteins affect the viroplasm development. We have found that a single ZIKV protein was sufficient to assemble a viroplasm-like structure complete with a toroidal shape surrounding the centrosome/Golgi MTOC complex.

P1953/B196

Superresolution Microscopy Reveals Coupling between Mammalian Centriole Subdistal Appendages and Distal Appendages.

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The unique feature of the mammalian mother centriole is the existence of distal appendages (DAPs) and subdistal appendages (sDAPs) at its distal end. Specifically, the structure of sDAPs and their relationship with DAPs are less well understood despite their obvious presence. Here, we use super-resolution microscopy to map the molecular architecture of the sDAP protein complex. In addition to the ordered organization relatively consistent with the hierarchical relationship known from genetic studies, we find unexpected features, including two-layer arrangements of ODF2 and CEP89, with one layer associated with DAPs and the other layer associated with sDAPs. Depleting DAPs by CRISPR-Cas9 knock-out of CEP83 removes the distal layer of ODF2; while sDAP depletion by knocking out CEP128 removes the proximal layer of ODF2 and CEP89. Thus, these two proteins play dual roles in DAPs and sDAPs. Furthermore, DAP depletion relaxes the longitudinal occupancy of ninein to span over the space of potential DAP sites and sDAP sites, suggesting that sDAP localization is partially dictated by the structure of DAPs. sDAPs also set the distal border of γ-tubulin at the G0 phase, where γ-tubulins are confined around the mother centriole. Microtubules (MTs) broadly occupy space around the centrioles, with some extending from DAPs and sDAPs. Depleting sDAPs eliminates a subset of MTs originated from the location close to the sDAP region, providing direct evidence of the MT anchoring role of sDAPs. Together, super-resolution microscopy enables the structural and functional understanding of sDAPs, surprisingly illustrating the coupling between DAPs and sDAPs and assuring that DAPs and sDAPs are not entirely independent.

P1954/B197

Material Aging Causes Centrosome Weakening and Disassembly during Mitotic Exit during Mitotic Exit.

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Centrosomes are membrane-less organelles that must resist microtubule-mediated forces for mitotic chromosome segregation. During mitotic exit, however, centrosomes are deformed and fractured by those same forces, which is a key step in centrosome disassembly. How the mechanical properties of centrosomes are tuned during the cell cycle is not known. Here, we used optically-induced flow perturbations to determine the molecular basis of centrosome deformation resistance and fracture resistance in *C. elegans* embryos. Induced flows caused time-dependent deformation and eventual fracture of the centrosome scaffold. Centrosome resistance to these flows peaked in metaphase, then declined sharply in anaphase, ~150s prior to natural disassembly. This mechanical transition depended on PP2A phosphatase and correlated with the departure of PLK-1 (Polo Kinase homolog) and SPD-2 (Cep192 homolog) from centrosomes. Acute inactivation of PLK-1 or SPD-2 made the centrosome scaffold weak and brittle in metaphase and led to premature disassembly. In vitro, PLK-1 and SPD-2

protected centrosome scaffolds from force-induced disassembly, suggesting that they directly reinforce the centrosome scaffold. In anaphase, centrosomes undergo material aging, whereby they lose PLK-1 and SPD-2andtransition from a strong, ductile state to a weak, brittle state that enables centrosome disassembly. Thus, centrosome mechanical properties are tuned via compositional balance of PLK-1 and SPD-2 versus PP2A.

P1955/B198

Cell Polarity Dependent Centrosome Separation in the *C. Elegans* Embryo.

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In animal cells, faithful chromosome segregation depends on the assembly of a bipolar spindle driven by the timely separation of the two centrosomes. Here we took advantage of the highly stereotypical cell divisions in *C. elegans* embryos to identify new regulators of centrosome separation. We find that at the two-cell stage the somatic AB cell initiates centrosome separation later than the germline P1 cell. This difference is strongly exacerbated by the depletion of the kinesin-13 KLP-7/MCAK, resulting in incomplete centrosome separation at NEBD in AB but not P1. Our genetic and cell biology data indicate that this phenotype depends on cell polarity via the enrichment in AB of the mitotic kinase PLK-1, which itself limits the cortical localization of the dynein-binding NuMA orthologue LIN-5. We postulate that the timely separation of centrosomes is regulated in a cell type dependent manner.

P1956/B199

CPAP Is Essential for Centriole Biogenesis but Not for Their Maintenance and PCM Organization.

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Centrosomes are major microtubule organizing centers in human cells. They are comprised of a microtubule-based core, centriole, and a surrounding pericentriolar material (PCM). CPAP is a centrosomal protein which mediates centriole initiation, elongation and PCM recruitment, and is localized to three distinct centrosomal fractions. One fraction is localized in mother centriole lumen, the second on procentrioles, and the third in pericentriolar material. Here we explore the functional interdependency of three different fractions of CPAP during centriole initiation, elongation, centriole disengagement and mitosis. We employ genetics and various modalities of microscopy including Stochastic optical reconstruction microscopy (STORM), to analyze the intra-centrosomal distribution and functional requirements of specific CPAP fractions. Using siRNA and an auxin-degradable system, we generated centrioles which lacked specific fractions of CPAP, allowing us to analyze them separately. We found that three CPAP fractions localize to the centrosome independently and are functionally independent. We find that neither PCM nor mother-centriole luminal CPAP fraction is needed for the process of procentriole duplication, elongation and disengagement. In addition, the removal of the PCM CPAP fraction does not affect the localization of inner and outer PCM components during interphase and in mitosis. Further, cytosolic and centrosome-dependent microtubule nucleation activity and mitotic progression were not perturbed in the absence of the PCM component of CPAP. Finally, a chronic depletion of CPAP to ~5% of its control physiological level appeared inconsequential for the maintenance of mature centrioles and centrosomes. In summary, we show functional independency of three centrosomal CPAP fractions, which corroborate the original reports suggesting that CPAP in

human cells is dispensable for the assembly of mitotic centrosomes but is critical for centriole structuring.

P1957/B200

Characterizing the Role of Mps1 and Erk in MAPK Mediated Centrosome Amplification.

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The MAPK signaling pathway is a phosphorylation cascade, consisting of RAS, RAF, MEK, and ERK, and is involved in a number of cellular processes including cell proliferation, differentiation, and growth. A number of constitutively active (CA) MAPK mutations have been identified in many cancers. However, the role of the MAPK proteins in the centrosome cycle is not well understood. Centrosome amplification has been shown to lead to genomic instability, both of which are common hallmarks of cancer. Previous work has shown that cancers containing CA B-RAF mutations contain supernumerary centrosomes and overexpression of CA B-RAF results in centrosome amplification. Additionally, CA BRAF dependent centrosome amplification has been linked to stabilization of a protein called Mps1, a dual functionality kinase that has roles in both the mitotic checkpoint and centrosome cycle. It has been demonstrated that MAPK signaling is important for localization of Mps1 at the mitotic checkpoint; however, less is known about how MAPK signaling interacts with Mps1 at the centrosome. We therefore aim to further characterize the relationship between Mps1 and MAPK signaling to better understand how supernumerary centrosomes form in cancer cells. To address this question, we assessed the localization of MAPK proteins, identified protein-binding partners, and generated mutations to increase the activity of MAPK proteins and assessed their effect on centrosome amplification. We have found that phospho(p)- and total ERK localizes to centrosomes in both interphase and mitotic cells and that localization is increased under prolonged S-phase arrest. Further, the amount of pERK accumulated at centrosomes increased more than two-fold when arrested in prolonged S-phase. Mps1 and ERK1/2 show reciprocal co-immunoprecipitation (IP), but MEK and B-RAF do not co-IP with Mps1. In order to explore the relationship between ERK and Mps1 on centrosomes, a mutant version of ERK, ERK L75P, S153D, demonstrating increased phosphorylation activity was generated. ERK L75P, S153D caused an increase in centrosome amplification over wildtype ERK in an Mps1-dependent manner. In conclusion, we have found that (p)ERK localizes to centrosomes, which is enhanced during a prolonged S-phase. ERK binds to Mps1 and ERK L75P, S153D causes Mps1-dependant centrosome amplification. This work greatly adds to the mechanistic understanding of how the MAPK pathway contributes to centrosome amplification in cancer. Further, given that our findings suggest that Mps1 is essential for MAPK induced centrosome amplification, Mps1 may be used as a potential therapeutic target in treating patients with activating mutations in the MAPK pathway.

P1958/B201

Disruption of Centriolin Impairs Differentiation in Rhabdomyosarcoma Cells.

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Rhabdomyosarcoma is the most common soft tissue cancer in children, arising from defects in myoblast differentiation. Previous studies have suggested that this differentiation process may be controlled by primary cilia, structures in the myoblasts that arise from the centrosome. However, it is not clear what factors in the primary cilia are required for the regulation of this process. Centriolin is an integral

component of both the centrosome and the primary cilia, suggesting that this protein may play an important role in myogenesis. In an attempt to identify centriolin's role in differentiation of rhabdomyosarcoma cells, the CRISPR/Cas9 gene editing system was used to eliminate the centriolin gene in cultured rhabdomyosarcoma cells and normal skeletal muscle cells, and the ability of these cells to differentiate was assayed. Immunofluorescence staining with antibodies against myosin heavy chain was used to indicate differentiation, and centriolin antibody was used to verify loss of this protein. Initial findings suggest that centriolin may play an essential role in differentiation, as disruption of centriolin leads to fewer cell aggregates and the absence of myogenic morphological characteristics, both indicators of ineffective differentiation. To determine the fate of the cells following loss of centriolin, Ki67 levels were examined. Ki67 levels were determined to be lower in both rhabdomyosarcoma cells and normal skeletal muscle cells lacking centriolin, indicating these cells are arrested and that this may play a role in the cells' inability to properly differentiate. Future studies are aimed to identifying the molecular mechanisms underlying centriolin's role in regulating this process.

P1959/B202

Emerging Role of Genetic Modifiers in the Etiology of Primary Microcephaly.

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Introduction Primary microcephaly (PM) is a neurodevelopmental defect characterized by a reduction in the cerebral cortex and mild intellectual disability (ID). The incidence for PM is 1/10,000 depending on ethnicity and the rate of consanguinity. There are approximately 25 genes known to cause PM. Besides small cerebral cortex and ID, several studies also report on the severe phenotypes of PM like short stature, extreme ID, speech impairment, salivation and seizures. But the underlying molecular mechanism for severity of such phenotypes is still unexplained. Up to now, little attention has been paid to genetic modifiers in case of PM that could be the culprit for severity of PM phenotypes. To address this question, we recruited families with PM from Pakistan. In our cohort, some of the families show significant interfamilial phenotypic variability. Here we report on a PM family with affected members in two different loops. Affected members of both loops of the same family differ in their phenotypes. Affected siblings in loop 2 show reduced height, severe ID, salivation, and speech impairment along with PM, but the patients in the loop 1 have only PM. Therefore, in this study we aim to unravel the novel role of genetic modifiers in causing the severity of phenotype in PM **Results** Sequencing of two patients each from both loops with customized gene panel "Mendeliome" (TruSight One gene panel from Illumina) demonstrated a novel homozygous *CENPJ* mutation (c.3586G>A; p.Asp1196Asn) in affected siblings of both loops and a reported heterozygous mutation of *PCNT* (c.5767C>T; p.Arg1923*) underlying microcephalic primordial dwarfism detected only in patients of loop 2 suggesting a role for *PCNT* as a genetic modifier. This data was further supported by observing severe cellular phenotypes like abnormal numbers of centrioles, large centrosome-nucleus distance, fragmented/supernumerary centrosomes as well as abnormal shape of nucleus, disorganized microtubules networks and reduced amount of CENP-J as well as PCNT detected by both immunofluorescence and western blotting in primary fibroblasts derived from patient of loop 2 only. Whereas, fibroblasts of patient from loop 1 did not show the cellular phenotypes observed in loop 2 except with reduced amount of CENP-J and

abnormal number of centrioles but less severe as compared to the cells from loop 2. Therefore, we propose that genetic factors may contribute to modify the severity of the *CENPJ* phenotype and, although based on suggestive cellular evidence, *PCNT* could function as one of such factors **Perspectives** This research could provide scientific community with new insights for consideration of genetic factors when analyzing the data into account for PM. In addition, it could also help in delineating the underlying pathways in the etiology of PM

P1960/B203
Microtubule-dependent Cytoplasmic MTOCs are Necessary for Spindle an choring and Ppositioning During Oocyte Meiosis**.**
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During oocyte meiosis I (MI), the spindle is assembled at a central position prior to its timely migration towards the cortex. Such asymmetrical positioning of the spindle following migration is crucial to extrude a small polar body (PB) and thereby retain a large proportion of the cytoplasm necessary to support early embryonic development. Remarkably, how these critical events-— spindle positioning and migration—are regulated during MI remains controversial and largely unknown. In mitotic cells, spindle positioning relies on centrosome-mediated astral microtubules (MTs). Interestingly, mammalian oocytes lack classic centrosomes and instead contain numerous MT organizing centers (MTOCs), whose only known function is to assemble the spindle. Whether MTOCs have other functions during MI is largely unknown. We fluorescently labeled MTOCs using AURKA-GFP or Cep192-GFP followed by time-lapse confocal microscopy. Interestingly, we observed two different sets of MTOCs: those that are known to form spindle poles (polar MTOCs; pMTOCs) and those that remain free in the cytoplasm (cytoplasmic MTOCs; cyMTOCs). The role of cyMTOCs during MI is unknown. We confirmed that these fluorescently-labeled structures in the cytoplasm are indeed MTOCs as evidenced by their co-immunostaining with γ-tubulin and their ability to nucleate MTs when exposed to taxol. Using time-lapse confocal microscopy, we found that pMTOCs have similar kinetics and behaviors to the cyMTOCs; suggesting that the latter might connect to pMTOCs. Importantly, using super-resolution microscopy, we found that cyMTOCs are connected to both the oocyte cortex and pMTOCs (at spindle poles) through MT connections suggesting that cyMTOCs anchor pMTOCs to regulate spindle positioning. To investigate this hypothesis, we used 2-photon laser ablation to deplete cyMTOCs followed by time-lapse confocal imaging of live oocytes. Strikingly, depletion of cyMTOCs results in severe defects in spindle positioning and spindle pole integrity. Our results suggest a novel model for spindle positioning during MI, in a large cell with short astral MTs, where MTs emanating from pMTOCs attach to cyMTOCs; which in turn act as amplifying sites for MT nucleation, anchoring pMTOCs to the cell cortex.

Spindle Assembly 2

P1961/B204

M2I-1, an Inhibitor of CDC20 and MAD2 Interaction, Increases Cancer Cell Lines Sensitivities to Anti-mitotic Drugs through Mcl-1s Pathway.

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Background: Drugs such as taxanes, epothilones, and vinca alkaloids are widely used in the treatment of breast, ovarian, and lung cancers but come with major side effects such as neuropathy and loss of neutrophils and as single agents have a lack of efficacy. M2I-1 (MAD2 inhibitor-1) has been shown to disrupt the CDC20-MAD2 interaction, and consequently, the assembly of the mitotic checkpoint complex (MCC). **Results**: We report here that M2I-1 can significantly increase the sensitivity of several cancer cell lines to anti-mitotic drugs, with cell death occurring after a prolonged mitotic arrest. In the presence of nocodazole or taxol combined with M2I-1 cell death is triggered by the premature degradation of Cyclin B1, the perturbation of the microtubule network, and an increase in the level of the pro-apoptotic protein MCL-1s combined with a marginal increase in the level of NOXA. The elevated level of MCL-1s and the marginally increased NOXA antagonized the increased level of MCL-1, a pro-survival protein of the Bcl-2 family. **Conclusion**: Our results provide some important molecular mechanisms for understanding the relationship between the mitotic checkpoint and programmed cell death and demonstrate that M2I-1 exhibits antitumor activity in the presence of current anti-mitotic drugs such as taxol and nocodazole and has the potential to be developed as an anticancer agent.

P1962/B205

Klp2 and Ase1 Synergize to Maintain Meiotic Spindle Stability during Metaphase I.

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The spindle apparatus segregates bi-oriented sister chromatids during mitosis but mono-oriented homologous chromosomes during meiosis I. It has remained unclear if similar molecular mechanisms operate to regulate spindle dynamics during mitosis and meiosis I. Here, we employed live-cell microscopy to compare the spindle dynamics of mitosis and meiosis I in fission yeast cells and demonstrated that the metaphase spindle length is ~two-fold longer in meiotic I cells than in mitotic cells and that the spindle elongates faster during meiosis I than mitosis. We further demonstrated that the conserved kinesin-14 motor Klp2 plays a specific role in maintaining metaphase spindle length during meiosis I, but not during mitosis. Moreover, the maintenance of metaphase spindle stability during meiosis I requires the synergism between Klp2 and the conserved microtubule crosslinker Ase1 as the absence of both proteins causes exacerbated defects in metaphase spindle stability. The synergism is not necessary for regulating mitotic spindle dynamics. Hence, our work reveals a new molecular mechanism underlying meiotic spindle dynamics and provides insights into understanding differential regulation of meiotic and mitotic events.

P1963/B206

One Stone Two Birds-Regulation of Cell Division by Nuclear Transport Factors.

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In conjunction with transport factors such as Importin-α/-β, the small GTPase Ran plays a crucial role in the delivery of macromolecules between the nucleus and cytoplasm during interphase. Importantly, assembly of the mitotic spindle is also orchestrated by the Ran pathway. Ran functions by modulating the interaction between transport factors and the spindle assembly factors (SAFs) that control many aspects of microtubule behavior, such as microtubule nucleation, stabilization and bundling. First, our work provides molecular details of how transport factors regulate the NuMA, one of SAFs, functioning facilitating assembly of higher-order microtubule structures, further illuminating how Ran-governed transport factors regulate diverse SAFs and accommodate various cell demands. Secondly, we further report a Ran pathway-independent attenuation mechanism that allows transport factor, Importin-α, to suppress the vesicle fusion mediated by p115 (a vesicular tethering factor) and required for mitotic Golgi disassembly. During early mitosis, Ran releases Importin-α from SAFs (e.g. NuMA), activating the mitotic functions of these SAFs and in turn promoting mitotic spindle assembly. Thus, the Importin-α liberated by RanGTP can instead interact with a Golgi associated protein, GM130, thereby suppressing p115-mediated vesicle-Golgi fusion and allowing Golgi disassembly. Altogether, our findings illuminate that the Ran and kinase-phosphatase pathways regulate multiple aspects of mitosis coordinated by nuclear transport factors (e.g. spindle assembly, Golgi disassembly).

P1964/B207

A Mitotic Screen in Indian Muntjac Cells Reveals a Role for Augmin in Kinetochore Fiber Maturation.

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Accurate chromosome segregation during mitosis is essential for life and relies on the activity of hundreds of proteins. However, due to technical limitations or limitations of the available model systems, some of their functions might have been overlooked. Here we propose to establish a unique placental mammal model system that combines the powerful genetic tools and low chromosome number of fission yeast and Drosophila melanogaster, with the exceptional cytological features of a rat kangaroo cell. This system is based on hTERT-immortalized fibroblasts from a female of the Indian Muntjac, a small deer which has the lowest known chromosome number (n=3) in mammals. Due to centromere-telomere and centromere-centromere tandem fusions during evolution, Indian Muntjac chromosomes are large and morphologically distinct, with one pair of acrocentric chromosomes (chromosomes 3+X) containing an unusually large compound kinetochore. Here we used an siRNA library to perturb the function of mitotic proteins in this system and investigated the respective mitotic phenotypes using spinning-disk confocal live-cell recordings and super resolution STED microscopy. With these approaches, combined with western blot analysis, we were able to confirm and investigate 56 RNAi phenotypes. The vast majority revealed that the molecular landscape required for mitosis is

conserved with other placental mammals, including humans, validating the Indian muntjac as a powerful model system. However, due to some specific features associated with the large kinetochore size of Indian muntjac, we uncovered a role for the Augmin complex, which has been implicated in microtubule-dependent microtubule amplification, in k-fiber maturation. This project contributes with a detailed phenotypic analysis of mitosis in Indian muntjac, offering a powerful resource open to the cell division community. Ultimately, this systematic analysis discloses overlooked functional roles for known mitotic proteins, while opening questions that were not addressable in more complex eukaryotic model systems.

P1965/B208

PLK1 Is Required for Normal Chromosome Compaction and Microtubule Organization in Mouse Oocytes.

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Errors in meiosis can result in genetic mutation and chromosome missegregation. Mammalian meiosis is sexually dimorphic and presents unique temporal challenges for oogenesis. Oocytes initiate meiosis during embryogenesis, then enter a prolonged late prophase (dictyate) arrest. Immediately prior to ovulation, oocytes will undergo nuclear envelope breakdown, chromosome condensation, and then homologous chromosomes segregate to complete meiosis I. Therefore, oocytes remain arrested at dictyate for long periods of time, which varies depending on when an oocyte is matured for ovulation. It is critical for oocytes to maintain their capacity to condense and segregate their chromosomes during dictyate arrest, but it is known that these events can be compromised, particularly in oocytes that have remained arrested for longer periods of time. Several cell cycle kinases have been linked with roles in coordinating events during meiotic resumption, including polo-like kinases (PLK). Mammals express four kinase-proficient PLKs, (PLK1-4). Studies assessing the role of mammalian PLK1 have previously relied on RNA knockdown and kinase inhibition approaches, as *Plk1* null mutations are embryonically lethal. To further study the role of PLK1 in mammalian meiosis, we have developed a *Plk1* conditional knockout mouse line. This novel genetic model specifically mutates *Plk1* in during meiotic prophase I, prior to the dictyate arrest. *Plk1* conditional knockout females are infertile, despite having a normal number of oocytes that successfully undergo nuclear envelope breakdown. A significant portion of these oocytes fail to maintain chromosome compaction, with defective cohesin and condensin localization. At the point of metaphase I, *Plk1*-depleted oocytes form either a monopolar microtubule organizing center or an abnormally small bipolar spindle, with aberrant localization of microtubule organizing center components. We conclude that PLK1 is essential for the post-resumption stages of oogenesis.

P1966/B209

Whole Exome Sequencing Implicates *CEP120* in Human an euploidy Conception Risk.

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Human infertility is a significant public health problem in the world that affects women, men and couples. In United States, surveys estimate that around 10-15% of the population struggle with infertility. In females, around the half of infertility cases are due to distinct phenotypes (i.e., physical abnormalities, polycystic ovarian syndrome, Perrault syndrome, etc), and at least 35% of cases have an

underlying genetic basis. Thus, determining the genetic drivers of infertility is important for a greater insight into the origins of this issue. The key event important for human reproduction is Meiosis, a specialized type of cell division producing female and male gametes through segregation of homologous chromosomes and sister chromatids during two subsequent meiotic divisions. Compared to male meiosis, female meiosis is highly error prone. Mis-segregation of chromosomes leads to the formation of around 20% of aneuploid eggs in young females and this rate drastically increases with advanced maternal age. Although advancing maternal age is an established contributing factor, some women undergoing in *vitro* fertilization (IVF) procedures have extreme aneuploid conception rates for their age. These observations indicate that other factors, such as maternal genetic variants, also contribute to infertility, irrespective of age. To identify genetic variants that could affect female egg quality we performed whole-exome sequencing on women that produced a high proportion of aneuploid blastocysts as determined by preimplantation genetic testing for aneuploidy following IVF. In these women, we identified an enrichment of coding variants of genes involved in cytoskeleton and microtubule formation. Using a mouse oocytes model, we validated the biological significance of a variant rs2303720 within the *Centrosomal protein 120* (CEP120) during meiotic maturation. We found that ectopic expression of CEP120 p.Arg947His caused decreased spindle microtubule nucleation efficiency and increased the incidence of aneuploidy at metaphase II. Importantly, this *CEP120* variant encodes a protein that has a dominant negative function. This study is the first step in identifying genes and mutations contributing to the risk of maternal aneuploidy and could allow prescreening of patients that have better chances to benefit from preimplantation genetic testing.

P1967/B210

Phospho-dependent Regulation of Competitive Interactions among Assembly Factors during Centriole Duplication in *C. Elegans*.

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Centrioles are microtubule-based organelles that aid in bipolar spindle formation and serve as basal bodies for nucleating cilia. Centriole duplication is a tightly controlled event in dividing cells that happens only once each cell cycle and mis-regulation of this process has been linked to diseases such as cancer and primary microcephaly. Studies in *C. elegans* have identified a set of core conserved centriole proteins: SPD-2, ZYG-1, SAS-5, SAS-6, and SAS-4. The overall theme of centriole assembly is conserved across genera, with only minor variations. Although general details of the assembly process have been worked out, the molecular aspects of the process are still not completely understood. The factors that trigger centriole duplication, the critical substrates of the kinase ZYG-1, and the importance of substrate phosphorylation are some of the intense areas of research. Unfortunately, difficulty in procuring recombinant core centriolar proteins in soluble form has hampered in *vitro* studies designed to address these questions. We here show that ZYG-1, SAS-6, SAS-5 and SAS-4 can be obtained in their functional form from *E. coli*. Although each of the proteins were insoluble initially, we were able to efficiently refold them in *vitro* under standardized conditions. Based on biophysical and functional studies, these proteins have attained their native conformation. Using the recombinant proteins, we discovered a novel interaction between ZYG-1 and SAS-5 and mapped the regions necessary for their binding. We find that while ZYG-1 can phosphorylate SAS-4, -5, and -6 in vitro, SAS-5 appears to be the preferred substrate. Further we find that many of the phosphorylated residues in SAS-5 are conserved among nematodes. Of particular interest, some of these residues are part of a larger conserved motif in the N-

terminus. Surprisingly, we found that this motif is required to mediate interaction of SAS-5 with both SAS-4 and ZYG-1, and that the precise pattern of phosphorylation within this domain, dictates the choice of ZYG-1 or SAS-4 as a binding partner. Using Cas9-mediated mutagenesis, we show that blocking phosphorylation of these residues results in sporadic centriole duplication failure and embryonic lethality. We hypothesize that phosphorylation of SAS-5 by ZYG-1 within this motif is necessary to specify a mutually exclusive mode of interaction of SAS-5 with ZYG-1 and SAS-4, and that this phosphorylation-dependent interaction contributes to the fidelity of centriole duplication.

P1968/B211

From Parts to Processes: in *Vitro* Reconstitution of Branched Microtubule Nucleation.

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Eukaryotic cell division requires the mitotic spindle, a microtubule (MT)-based structure which accurately aligns and segregates duplicated chromosomes. The dynamics of spindle formation are determined primarily by correctly localising the MT nucleator, γ-Tubulin Ring Complex (γ-TuRC), within the cell. A conserved MT-associated protein complex, Augmin, recruits γ-TuRC to pre-existing spindle MTs, amplifying their number, in an essential cellular phenomenon termed “branched” MT nucleation. Here, we purify endogenous, GFP-tagged Augmin and γ-TuRC from *Drosophila* embryos to near homogeneity using a novel one-step affinity technique; **cl**eavable **A**ffinity **P**urification (cl-AP). We demonstrate that, in *vitro*, while Augmin alone does not affect Tubulin polymerisation dynamics, it stimulates γ-TuRC-dependent MT nucleation. We also show the combined nucleating activity of mitotic Augmin-γ-TuRC is greater when complexes are isolated from mitotic, rather than cycling cells, suggesting this phenomenon is regulated in *vivo in* a cell cycle-dependent manner. We also assemble and visualise the MT-Augmin-γ-TuRC-MT junction using light microscopy, conclusively demonstrating the polarity-specific nature of the interaction. Our work therefore reconstitutes branched MT nucleation. It also provides a powerful synthetic approach with which to investigate the emergence of cellular phenomena, such as mitotic spindle formation, from component parts.

P1969/B212

The Value of Drawing in Scientific Practice: New Representations of Cell Division and Process Epistemology.

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Modern scientific practice reflects the current value the research community places on quantifying biological (cellular) phenomena and in representing it within a mechanical framework. However, the philosophical thesis that all living systems should be envisaged dynamically raises pressing questions about how they can be best represented, qualitatively. Here, we describe a collaborative project between a philosopher of biology, an artist and a cell biologist, focusing on the process of cell division. A series of ‘Drawing Labs’ and one-to-one sessions between the artist, the cell biologist and their research group, designed to uncover implicit and accumulated understanding, and reinforced by discussion with the philosopher, has resulted in the generation of drawings which attempt to represent the whole process of cell division in one connected image. This, in turn, has led us to reappraise the value of drawing in biological practice. We have begun to reincorporate observational, experiential and imaginative exercises and practices within microscopy-based methodologies, arguing that a more

holistic scientific approach can yield new knowledge of those processes and stimulate new and relevant hypotheses.

P1970/B213

Dual Spindle Assembly in Mammalian Zygotes Is Independent of the Presence of Centrioles.

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Dual spindle assembly in mammalian zygotes is independent of the presence of centrioles in mammals, the genetic material from each parent is replicated in a separate pronucleus after fertilization of the egg. For a long time, it was unknown why the two sets of parental chromosomes still occupy separate compartments when united in one nucleus after the first division of the zygote. Recently, we discovered that in the first murine embryonic mitosis two individual bipolar spindles form around each zygotic pronucleus, which independently congress chromosomes during pro-metaphase and only later align to segregate the two parental genomes in parallel. The formation of exactly two bi-polar spindles in one cell furthermore suggested that spindle assembly is not entirely driven by self-organization of many randomly distributed cytoplasmic microtubule organizing centers (MTOCs) as in the oocyte, but that chromosomal nucleation is likely to play an important role in the zygote. Before defining this mechanism further, we wondered if dual spindle assembly and the chromosomal nucleation pathway are indeed generally relevant for mammals. This is especially important to address in non-rodent species where zygotes inherit centrioles from the sperm and thus have only two MTOCs in contrast to the many present in murine embryos. To answer this question, we set up the in *vitro* maturation and fertilization of oocytes and mRNA microinjection to express GFP marker proteins in zygotes in the bovine model system, where two centrioles are inherited from the sperm like in humans. We then imaged the behavior of chromosomes and microtubules throughout the first mitotic division using an inverted light-sheet microscope developed in the group. Very interestingly, we found that despite the presence and incorporation of centrioles into the spindle, also in bovine zygotes two separate microtubule arrays frequently form around the pronuclei. Taken together, these results show that dual spindle formation is conserved both in species with multiple or only two MTOCs, and makes it very likely that a microtubule nucleation pathway originating from chromosomes plays an important role in the first embryonic division of mammals in general. By perturbing microtubule polymerization at the onset of spindle formation in mouse zygotes, we have indeed found evidence of a chromosomal nucleation pathway. We will present new data, where we further explore the nature of this nucleation as a key process that ensures faithful chromosome segregation at the beginning of mammalian life.

P1971/B214

Air Pollution and Chromosomal Segregation: the Effect of Particulate Matter in the Spindle Assembly Checkpoint, Trough Overexpression of the Long Non-Coding RNA *NORAD*.

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Background: Air pollution represents a worldwide problem, impacting in human health predominantly in major cities, where traffic or industry activities releases gases and solid particles in the air. Particulate matter of 10 micrometers or less in diameter (PM_{10}) is considered as an agent related to cardiopulmonary diseases, including lung cancer. Chromosomal segregation is controlled by the long

non-coding RNA (lncRNA) *NORAD*, as well as by spindle assembly checkpoint (SAC), in which proteins such as MAD1, MAD2, BUBR1, AURORA B and SURVIVIN orchestrate a mitosis-delay signal in order to ensure genomic instability. Alterations in SAC causes aneuploidy, a feature associated to carcinogenesis. Although PM_{10} are associated to the generation of chromosomal alterations, the impact in chromosomal segregation mechanisms has not been characterized. The **aim** of this study is to evaluate the effect of PM_{10} in the expression of the lncRNA *NORAD*, as well as the effect in the expression of SAC genes and in the control of chromosomal segregation/mitosis, using the A549 cell line (lung cancer) as our model. **Materials and methods:** Synchronized A549 cells were exposed to PM_{10} (10 µg/cm^2) for 24 h to evaluate the expression of the genes *NORAD, MAD1L1, MAD2L1, BUB1B, AURKB y BIRC5* by RT-qPCR. To serve as controls of SAC activation, cells were exposed to taxol (100nM) and doxorubicin (0.25 µg/mL). Finally, *NORAD* was inhibited by siRNAs in all treatments. **Results:** PM_{10} increases the expression of *NORAD* (53%), MAD1L1 (61%), MAD2L1 (48%), BUB1B (36%), AURKB (41%) and BIRC5 (37%) in A549 cells, compared with non-treated cells ($p<0.05$). Taxol caused an increase in mRNA levels of these genes, while doxorubicin caused only an increase in *NORAD* expression (188%). *NORAD* inhibition decreased the expression of *MAD1L1, MAD2L2 y BUB1B* in presence of PM_{10}, taxol or doxorubicin. This effect was not seen in AURKB y BIRC5. **Conclusion:** Particulate matter PM_{10} induces overexpression of the lncRNA *NORAD*, as well as the overexpression in SAC components (*MAD1L1, MAD2L2 y BUB1B*), predisposing to the generation of aneuploid cells.

P1972/B215

Myl5 Associates with the Mitotic Spindle and Is Required for Cell Division.

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Cell division is a highly regulated process that ensures the proper distribution of genetic material to daughter cells. The mitotic microtubule spindle is a structure that is critical for chromosome congression and chromosome segregation, which is important for the fidelity of cell division. We recently performed a screen for mitotic microtubule interacting proteins and identified a Novel Myosin Light Chain 5 (Myl5) as a putative mitotic microtubule binding protein. Here, I present data on the characterization of Myl5 and its role in cell division. Myl5 is a myosin regulatory light chain that is upregulated in late stage cervical cancer patients and has been shown to promote metastasis. I discovered that Myl5 is essential for mitotic spindle assembly and proper cell division. Myl5 localizes to the spindle poles during mitosis and co-localizes with spindle pole proteins and Myo10 in a cell cycle dependent manner. Depletion of Myl5 led to spindle assembly defects and lagging chromosomes. Additionally, the localization of Myl5 to the spindle poles depended on microtubules and Myo10. These results elucidate a novel function for Myl5 in early spindle assembly and chromosome segregation. I have proposed a model where Myl5 is a component of the Myo10 complex, which functions to ensure the fidelity of chromosome segregation. This study is the first to highlight how Myl5 misregulation could drive carcinogenesis.

P1973/B216

Distinct Modes of Chromosome Lagging in Meiosis-i Predict an euploidy in Mouse Oocytes.

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The incidence of chromosome segregation errors that cause aneuploidy in the first meiotic division (MI) increases with age and is considered a major contributing factor of age-related decline in female fertility. A frequently observed segregation defect in aged oocytes is lagging anaphase chromosomes,

though the extent to which these directly contribute to aneuploidy in mouse oocytes is unclear. To understand the importance of lagging chromosomes in generation of aneuploidy, we combined live and fixed confocal fluorescence microscopy to monitor chromosomal behavior and examine their attachments to spindle-microtubules during MI. Live imaging revealed an increased incidence of lagging chromosomes during anaphase in old versus young oocytes (58.8% vs. 26.7%). Surprisingly, not all lagging chromosomes had common origin and exhibited identical behavior. Rather, we identified two distinct types of lagging chromosomes that we refer to as 'canonical', that originated from aligned bivalents, and 'non-canonical' that originated from mildly misaligned bi-oriented bivalents. Importantly, the presence of 'canonical' lagging chromosomes, reminiscent of those found in mitotic cells, strongly correlated with aneuploidy outcome of anaphase, while the presence of 'non-canonical' ones showed no correlation. Interestingly, the examination of chromosome attachment status shortly before anaphase revealed that in both young and old oocytes, the proportion of incorrect/merotelic attachments, considered responsible for producing lagging chromosomes, was negligible. However, the proportion of chromosomes lacking stable microtubule attachments remained constantly high (20-30%) throughout entire MI in old oocytes. At the onset of anaphase, all chromosomes in both groups become stably attached to microtubules, suggesting that a large proportion of chromosomes in old oocytes rapidly stabilize their attachments just prior to anaphase. We propose that this sudden formation of stable microtubule attachment might be highly error-prone thus giving rise to 'canonical' lagging chromosomes that would be responsible for aneuploidy in old oocytes.

P1974/B217

S6k1 Drives Polycystic Kidney Downstream of Mtor by Perturbing Oriented Cell Division.

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mTOR activation is essential and sufficient to cause polycystic kidneys, as observed in Tuberous Sclerosis Complex (TSC) and other genetic disorders. However, the mechanistic targets of mTOR responsible for cyst formation remain to be established. In disease models, a sharp increase of proliferation and cyst formation correlates with a dramatic loss of oriented cell division (OCD). Surprisingly, combining mouse models and 3D kidney epithelial cells culture, we find that OCD distortion is intrinsically due to S6 kinase 1 (S6K1) activation. The concomitant loss of S6K1, in *Tsc1* mutant mice, restores OCD but does not decrease hyperproliferation, leading to non-cystic harmonious hyper growth of kidneys. mTOR/S6K1 hyperactivity alters centrosome positioning in mitotic cells. Mass spectrometry-based phosphoproteomics for novel S6K1 substrates reveals targets involved in cell adhesion and the actomyosin cortex. Among them is Afadin, a known component of cell-cell junctions required to couple intercellular adhesions and cortical cues to spindle orientation. Afadin is directly phosphorylated by S6K1 and abnormally accumulates with E-cadherin at the apical surface in *Tsc1* mutant cells. Phospho-mutant Afadin rescues sphere formation in *Tsc1 and Afadin* knockout cells. By revealing two distinct branches regulating the rate and orientation of cell division downstream of mTOR, our data may reconcile the need of both cellular alterations at the onset of cystogenesis.

P1975/B218

A *Drosophila* Gene, Cg10126, Is Required for Normal Mitotic Spindle Elongation, Bipolarity and Chromosomal Stability.

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Our lab is interested in epidermal growth factor receptor (EGFR)-directed cell proliferation and differentiation in developing *Drosophila*. In a screen for transcriptional targets of EGFR activity, we identified a *Drosophila* gene, CG10126, that is upregulated 10-fold in response to EGFR activation. CG10126 is orthologous to human Calcyphosine (CAPS), whose expression is upregulated in many human cancers. Subsequent work from our lab showed that CG10126 is localized to the mitotic spindle and is required for normal levels of mitosis. From these observations, we hypothesized that CG10126 might be required for spindle stability and integrity. To test this hypothesis, we knocked down CG10126 using RNAi in cultured S2 cells followed by immunocytochemistry. We then compared spindle length and phenotypes between control and knockdown cells. During analysis, we observed a significant decrease in mitotic spindle length and an increase in the number of abnormal spindle phenotypes among cells with reduced CG10126 compared to control. These results suggest CG10126 is essential for mitotic spindle stability and integrity in *Drosophila* and suggests that CAPS might also affect spindle formation and mitosis in humans.

P1976/B219

Characteristics of Spindle Assembly Checkpoint Gene Expression.

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p.p1 {margin: 0.0px 0.0px 6.0px 0.0px; font: 11.0px 'Times New Roman'; color: #404040} the mitotic checkpoint or spindle assembly checkpoint (SAC) is a signaling pathway that safeguards proper chromosome segregation. The core of the checkpoint mechanism is a network of protein-protein interactions. Proper functioning of the SAC depends on adequate protein concentrations and appropriate stoichiometries between these proteins. This makes it important to understand the underlying gene expression and its quantitative parameters. Not much is known about SAC gene expression in any organism. We have systematically mapped genetic regions that are necessary for the expression of SAC genes in fission yeast (*S. pombe*). Short upstream regions are sufficient for wild type-like expression and may consist of a core promoter only. The *mad1* and *mad2* genes, coding for proteins forming a tight complex, have unusually short 5’ UTRs. Expression of SAC genes is additionally influenced by the coding sequence. This is partly through a high fraction of “non-optimal” codons, which lowers the steady-state mRNA concentration. As in budding yeast (*S. cerevisiae*), this requires the RNA helicase Ste13 (S.c. Dhh1), suggesting that the pathway linking codon optimality to mRNA stability is conserved between budding and fission yeast. Overall, our work extends the so far largely protein-centric view of SAC function to the underlying gene expression control.

P1977/B220

ATP Availability Limits Mitotic Duration and Impacts Cell Fate Determination.

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Little is known regarding the impact of energy metabolism on a prolonged mitosis and it remains unclear which processes are energy dependent under conditions that prevent SAC satisfaction (e.g. unattached kinetochores and/or in the presence of anti-mitotic chemotherapy drugs). Our data, from live cell imaging, shows that in different human cell lines mitotic energy content is a critical factor for the cell fate decision following a prolonged mitosis. We demonstrate that decreasing mitotic ATP levels, accompanied by increasing AMPK activation, leads to a switch in cell fate from mitotic cell death to slippage, thereby enhancing cell survival frequency. This increase in mitotic slippage results from a weakened SAC response, since cells treated with ATP synthesis inhibitors show a faster degradation of the mitotic substrate Cyclin B1. In agreement, inhibition of the anaphase-promoting complex/cyclosome (APC/C), the major regulator of Cyclin B1 levels during mitosis, rescues this phenotype, switching cell fate from mitotic slippage to death. We found that kinetochore localization of Mad1, CENP-E and Spindly was not significantly compromised, which suggests that the weakened SAC response was independent of kinetochore SAC signaling. Conversely, we found that *de novo* protein synthesis was decreased in energy deficient cells, and this was independent of AMPK signaling pathway. Importantly, CDC20 levels were significantly decreased which compromised mitotic checkpoint complex (MCC) production, thereby causing a premature activation of the APC/C and consequent mitotic slippage. In agreement with this, mitotic arrested cells treated with the global protein synthesis inhibitor cycloheximide phenocopy energy deficient conditions. Additionally, we found by mass spectrometry analysis and western blot analysis a global dephosphorylation of APC/C subunits 1 and 3 in energy deficient cells, suggesting a lower CDK1 activity. Critically, we demonstrated that APC3 requires continuous CDK1 dependent mitotic phosphorylation, which might contribute to strengthen SAC signaling. Overall, our data suggests that *de novo* ATP synthesis during a prolonged mitosis is required for continuous protein translation and CDK1 kinase activity. Our findings bring awareness to the limitations of using ATP deprivation as a therapeutic option in anti-tumoral strategies.

P1978/B221

Branching Microtubule Nucleation Is the Main Source of Microtubules Generated at Chromosomes in Meiotic *Xenopus* Egg Extracts.

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Microtubules are made in several places in the dividing eukaryotic cell to form the spindle. Though centrosomes are the most visually prominent hubs of microtubule nucleation in most cell types, microtubules also form at chromosomes during cell division. Indeed, chromosomal microtubules are necessary for assembling the entire spindle in cells that lack centrosomes, such as egg cells. Alongside centrosomes and chromosomes, microtubules themselves act as sites for generating new microtubules, a process referred to as branching microtubule nucleation. Chromosomal and branched microtubules are crucial for the proper alignment and segregation of chromosomes, and both rely on the GTPase RanGTP. RanGTP exists as a gradient centered around chromosomes and spurs the release of spindle assembly factors (SAFs). One of these SAFs is the protein TPX2, which stimulates branching microtubule nucleation together with the protein complex augmin and the γ-tubulin ring complex. It is currently not

understood how microtubule nucleation around chromosomes is related to branching microtubule nucleation. In this study, we investigated the relationship between chromosomal and branching microtubule nucleation. To do so, we developed an assay to watch microtubule nucleation and growth at chromosomes *ex vivo*. We attached chromosomes purified from mitotic cells to the coverslip of a microscope flow chamber and then introduced *Xenopus* egg extract. Using TIRF microscopy, we visualized that chromosomes generate branched microtubule networks. This can even occur from a single microtubule whose lattice directly overlaps or touches chromatin. These experiments define the distances and times at which branching microtubule nucleation is stimulated around chromosomes. Notably, we saw that a single microtubule captured at the kinetochores can also serve as the mother microtubule upon which daughter microtubules will nucleate. This finding helps explain how a kinetochore fiber can be made efficiently. Finally, by depleting TPX2 and augmin from the extract, we demonstrated that branched microtubules are the chief source of the microtubules that grow from chromosomes. Taken together, our experiments show that branching microtubule nucleation mediated by TPX2 and augmin is the main source of microtubules generated at chromosomes and kinetochores in *Xenopus* egg extracts. We propose that branching microtubule nucleation is the main pathway initiated at chromosomes to assemble spindles and capture kinetochores.

P1979/B222

Cellular Limits on the an aphase Spindle Elongation Rate Ensure That Chromosomes Are Dynamically Disentangled during Cell Division.

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At anaphase, elongation of the mitotic spindle facilitates the segregation of duplicated sister chromatids into future daughter cells. This elongation is driven by forces derived from the combined action of motor proteins and microtubule polymerization at the spindle midzone. In all known eukaryotes, a regulatory switch linked to the metaphase to anaphase transition greatly reduces the magnitude of force that is produced at the spindle midzone, thereby limiting the maximum speed at which the spindle elongates in anaphase. While the molecular correlates of this regulatory switch have been described in detail, the cellular consequences of exceeding this speed limit is unknown. In this study, we dramatically increased the speed of anaphase spindle elongation in budding yeast by introducing four point mutations into *Cik1*, a binding partner of the kinesin 14 motor protein Kar3. These mutations abrogate the interaction of the Kar3-Cik1 complex with the plus end microtubule tracking protein Bim1. Loss of this interaction leads to a ~3-fold increase in anaphase spindle elongation rate, without causing spindle collapse. We found that cells with these rapid anaphase spindle elongation rates were unable to properly disentangle separating chromosomes, leading to increased persistence, and more frequent breakage, of ultrafine DNA bridges. As a result, cells with rapid anaphase spindle elongation rates had increased incidence of DNA damage at anaphase, as well as delays in cytokinesis, ultimately leading to a ~6-fold increase in cells that were stalled in G2 with improper chromosomal content. Taken together, these findings describe a novel and critical protective function of the speed limit that cells impose upon the anaphase spindle elongation rate: the timing that is set by a tightly regulated anaphase spindle elongation speed is required to ensure that chromosomes are dynamically disentangled during anaphase chromosome segregation.

P1980/B223

Mechanisms of Acentrosomal Spindle Assembly and Maintenance in *C. Elegans* Oocytes.

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In female germ cells, or oocytes, the meiotic divisions are mediated by a microtubule-based spindle that is built and stabilized in the absence of centrosomes. While much is known about the proteins and forces underlying centrosome-based spindle assembly in mitotically-dividing cells, the molecular basis of force generation in acentrosomal spindle assembly and maintenance is far less understood. We are using *C. elegans* as an in *vivo* model to investigate these mechanisms. We previously characterized the stages of acentrosomal spindle assembly in *C. elegans* oocytes and found that KLP-18, a kinesin-12 family microtubule motor, and MESP-1, a rapidly evolving adaptor protein, are essential for outward force generation during this process. However, the biochemical mechanism of how these proteins generate force was unknown. We have now employed a combination of in *vitro and* in *vivo* approaches to gain insight into this important problem. First, we purified recombinant truncations of the KLP-18 coiled-coil stalk domain along with full length MESP-1 to use in microtubule binding experiments in *vitro.* We identified a novel non-motor microtubule binding site at the C-terminus of the KLP-18 stalk and found that this microtubule binding site is activated through MESP-1 interaction with an adjacent region of the stalk. Therefore, MESP-1 binding to KLP-18 may allow the motor to crosslink two microtubules using its motor and C-terminal microtubule binding domains, thereby generating force. To interrogate this model, we tested the importance of the KLP-18 C-terminal microtubule binding site in *vivo* using a temperature sensitive mutant strain containing two amino acid substitutions in the mapped domain. Prolonged incubation at the restrictive temperature caused spindle assembly defects that are identical to those observed following depletion of KLP-18 by RNAi; monopolar instead of bipolar spindles formed. In addition, we found that short incubation of this mutant at the restrictive temperature caused the collapse of already formed bipolar spindles into monopoles. In both cases, KLP-18 still localized to the aberrant spindles, indicating that the protein is present but non-functional. These results demonstrate that the C-terminal microtubule binding site that we identified in *vitro* is required for both spindle assembly and for the maintenance of spindle bipolarity in *vivo*. This work sets the basis for further investigation into how microtubule-associated proteins govern spindle assembly and maintenance, specifically in a system lacking centrosomes.

P1981/B224

Small Ovary Is Required for Genome Stability and Viability in Early*Drosophila*Embryos.

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Heterochromatin contributes to the regional inactivation of gene expression required for normal cell function and viability. We recently identified *Drosophila small ovary* (*sov*) as a novel player in heterochromatin stabilization. While null animals are inviable, partial loss-of-function mutations lead to female sterility associated with degenerate oogenesis. Germline-specific depletion of *sov* permits oogenesis, yet results in embryonic lethality, indicating a maternal contribution of *sov* is required. We examined the requirements for *sov in* embryo development. While *sov* mRNA is enriched at centrosomes, Sov protein localizes within the nucleus in a cell cycle-dependent manner, where it colocalizes with the essential heterochromatin factor, HP1a. Depletion of *sov* by germline-specific RNAi or through germline mutant clonal analysis results in mitotic asynchrony and DNA damage, indicating

Sov is necessary for genome stability. Live imaging reveals *sov* knockdown results in prolonged prophase, delayed chromosome congression, and errant chromosome segregation. Further, we demonstrate Sov is required for efficient microtubule organization, as loss of *sov* causes a range of spindle defects. Ultimately, these defects result in impaired cellularization and embryonic lethality. These findings suggest Sov is required for the fidelity of cell division during early embryogenesis.

P1982/B225

Dynein and ZYG-9 are required for acentriolar pole stability and maintenance in *C. elegans* oocytes.

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During cell division, a bipolar microtubule-based spindle is required to ensure faithful chromosome congression and segregation. While centrosomes function as microtubule organizing centers (MTOCs) to aid spindle assembly in most cell types, oocyte meiotic spindles of many species are acentriolar; how spindle poles form and are maintained in the absence of centrosomes is poorly understood. Some microtubule-associated proteins (MAPs) that localize to acentriolar poles have been previously identified; we are studying these proteins in *C. elegans* oocytes to determine if they have a role in meiotic spindle assembly and maintenance. First, we assessed DHC-1, a *C. elegans* homolog of the heavy chain of dynein, and found that long-term depletion of this motor using an auxin-inducible degron (AID) system resulted in splaying of microtubules at the poles and an increased number of disorganized spindles, suggesting a role in pole formation during spindle assembly. We also used *emb*-30 RNAi to induce metaphase arrest and then acutely depleted DHC-1 from pre-formed bipolar spindles. This resulted in similar phenotypes, suggesting that dynein is also required to maintain the integrity of poles after they form. We obtained similar results when we used the AID system to deplete another pole protein, ZYG-9, the *C. elegans* homolog of XMAP215. Following long-term depletion of ZYG-9, oocyte spindles did not stably achieve bipolarity, and poles went through a process of splitting and coming back together. Moreover, acute ZYG-9 depletion from metaphase-arrested spindles resulted in pole fragmentation, demonstrating a role for ZYG-9 in acentriolar pole stability and maintenance. Finally, we used the same AID approach to deplete DHC-1 and ZYG-9 from monopolar spindles (generated by *klp-18* RNAi), as another assay for pole integrity. Interestingly, in this context DHC-1 and ZYG-9 depletion had markedly different phenotypes. While depletion of ZYG-9 did not dramatically affect monopolar spindle morphology, DHC-1 depletion led to disintegration of the monopole, releasing chromosomes and associated microtubule bundles into the cytoplasm. Taken together, our data suggest that dynein and ZYG-9 are both required for acentriolar pole stability and maintenance during oocyte meiosis, but likely perform distinct functions. Further investigation of how these proteins stabilize acentriolar poles will help elucidate how mechanisms in meiotic spindles compare and contrast to those with centrosomes.

P1983/B226

Endoplasmic Reticulum (ER) Membranes Impose a Physical Constraint on the Mitotic Spindle.

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Chromosome capture, alignment and segregation all depend on the microtubule-based mitotic spindle, the major force generator inside cells. However, whether spindle function and checkpoint control are mechanically decoupled from the cytosol and cell cortex is still uncertain. In *Drosophila melanogaster* syncytial embryos, the ER is excluded from the spindle and forms an envelope with seemingly elastic

properties. The ER envelope surrounds the spindle throughout mitosis, but whether it is involved in the mechanics of chromosome segregation is not known. Conventional inactivation approaches are difficult to interpret owing to the importance of the ER for various cellular processes. To circumvent this experimental limitation, we disrupted this membranous organelle specifically, in a highly acute manner, using microinjection techniques for *D. melanogaster* syncytial embryos. We show that acute ER membrane fragmentation by ectopic addition of Reticulon-like protein 1 (Rtnl1) changes the surrounding environment and shortens spindle length over time in metaphase-arrested embryos. To probe for spindle function upon ER fragmentation we released the cohesive forces that hold chromosomes together and tested if the mitotic spindle can pull sister chromatids apart. Under these conditions spindle function is perturbed in multiple ways. Although chromosomes are pulled apart when the ER barrier is absent, chromosome movement occurs concomitantly with spindle elongation beyond the length under control condition. The oscillatory motions typically seen during alignment and under premature loss of cohesion are not observed. When the ER fragmentation is induced during interphase, one of the mitotic checkpoint proteins, Mad2, accumulates both along the spindle midzone and at the poles, leading to an apparent arrest of the cycle at metaphase. While this localization of Mad2 suggests that the checkpoint silencing mechanism is active, its mechanical framework is altered. Thus, our results reveal that the ER imposes a physical constraint on the mitotic spindle. Furthermore, they suggest that the ER mechanically feeds back to the mitotic checkpoint control. Ongoing experiments are now aiming to dissect the principles behind this action.

P1984/B227

3d Reconstruction of Meiotic Spindles in Mouse and Humans.

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Hindered by the limits of light microscopy we can neither resolve individual microtubules connecting to the chromosomes, nor their interaction with other components of the spindle. 3D Electron tomography provides the 'ultimate' resolution to the lack of structural information.We study the structure of individual chromosomes and their interactions with microtubules by 3D tomography, providing single microtubule resolution, and state-of-the-art light microscopy. Subsequently, computational analysis and simulations will be used to develop a mechanical model of how shape and structure of individual chromosomes affect their faithful segregation. Our methodology allows us to detect differences between individual chromosomes and their interactions with microtubules on a microscale level. We will present our preliminary results on the reconstruction of mouse meiotic spindles in Metaphase II.

P1985/B228

Probing the Dynamic Mitotic Proteome to Understand the Biological Clock Underlying the Mitotic Surveillance Pathway.

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Faithful replication and segregation of genetic information is essential for successful cell division. Indeed, numerous safeguards have evolved to prevent errors in these processes. Delays in cell division lead to an increased frequency of mitotic errors and DNA damage. Consequently, the Mitotic Surveillance Pathway (MSP) measures mitotic duration and triggers an arrest in cells that exceed a specific mitotic temporal threshold (*Sluder 2010*). Despite the important role of the MSP in protecting

genomic integrity, how cells determine mitotic duration remains unknown. We hypothesize that the shifting abundances of pro- and anti-arrest proteins over the course of normal mitotic progression is the mechanism for such a timer, but as of yet, no such proteins have been robustly linked to the failsafe pathway. Here, mass spectrometry analysis of highly synchronous mitotic lysates reveals the dramatic depletion of a subset of the mitotic proteome, generating a candidates list for drivers of the MSP timer and potentially identifying novel APC/C substrates. These proteins were further scrutinized for their possible role in the MSP as well as their mechanism of degradation. Finally, MSP proteins 53BP1, USP28, and p53, previously identified through genome wide CRISPR knockout screens, were used as a platform for further mechanistic understanding of the pathway.

Kinetochore Assembly and Functions 2

P1986/B229

***In Vitro* Reconstitution Unveils the Phospho-regulated Ska-Ndc80 Macro-complex.**

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Faithful chromosome segregation requires proper kinetochore-microtubule interactions during mitosis. The human Ska (spindle and kinetochore associated) complex is essential for mediating kinetochore-microtubule attachments and its loading onto the kinetochore complex Ndc80 (Ndc80C) plays a critical role in its function. However, the key determinant that docks Ska onto Ndc80C still remains elusive. Here, we address this question using biochemical reconstitution followed by functional analysis. We identified six Cdk1 sites in Ska3 distributed in three conserved regions, including the previously identified T358 and T360. In vitro, Cdk1 phosphorylation on the Ska complex enhanced WT, not phospho-deficient 6A, binding to Ndc80C. Strikingly, the phospho-mimetic Ska 6D complex formed a stable macro-complex with Ndc80C, but Ska WT failed to do so, suggesting that Cdk1 phosphorylation is necessary and sufficient for the formation of the Ska-Ndc80 macro-complex. In cells, Ska 6A completely lost its localization to kinetochores and its functions in chromosome segregation; whereas Ska3 6D partially restored them. Quadruple mutations that excluded T358 and T360 barely affected Ska3 localization at kinetochores and its functions; whereas, quadruple mutations that included these two sites significantly compromised Ska3 kinetochore recruitment as well as its functions. Altogether, our findings not only reveal the key phospho-regulated Ska-Ndc80 macro-complex essential for faithful chromosome segregation, but also pave the way for mechanistic analyses of kinetochore-microtubule interactions.

P1987/B230

Inter-kinetochore Tension Promotes Microtubule Release and Inhibits Depolymerization in Response to Aurora B Activity.

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Error-free chromosome segregation requires Aurora B kinase regulating the interaction between kinetochores and associated microtubules. Two mechanisms by which Aurora B resets incorrect

chromosome configurations have been proposed: inducing kinetochore-microtubule unbinding or inducing microtubule depolymerization while maintaining kinetochore attachment. To distinguish between these models, we acutely recruited Aurora B to kinetochores using a photo-activatable chemical dimerizer. Because kinetochore-microtubule unbinding relaxes inter-kinetochore tension whereas microtubule depolymerization generates tension, the contrasting mechanisms would cause opposite directions of chromosome movement. For a monopolar spindle, we find that Aurora B recruitment drives kinetochore poleward movement, consistent with Aurora B promoting microtubule depolymerization under low resisting loads. In contrast, Aurora B recruitment to a single kinetochore of a bioriented sister kinetochore pair can trigger movement of the targeted kinetochore away from its attached pole, indicating kinetochore-microtubule unbinding under high tension. Furthermore, increasing inter-kinetochore distance increases the likelihood of unbinding rather than microtubule depolymerization. Thus, the kinetochore response to Aurora B activity depends on the cellular context: depolymerization at low tension and unbinding at higher tension. These findings suggest distinct mechanisms to correct different microtubule attachment errors, under the control of a single kinase.

P1988/B231

Kinetochores Attached to Microtubule-ends Are Stabilised by Astrin Bound PP1 to Ensure Proper Chromosome Segregation.

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Microtubules segregate chromosomes by attaching to macromolecular kinetochores. Only microtubule-end attached kinetochores can be pulled apart; how these end-on attachments are selectively recognised and stabilised is not clear. Using the kinetochore and microtubule-associated protein, Astrin, as a molecular probe, we show that end-on attachments are rapidly stabilised by spatially-restricted delivery of PP1 near the C-terminus of Ndc80, a core kinetochore-microtubule linker. PP1 is delivered by the evolutionarily conserved tail of Astrin, which promotes Astrin's own enrichment creating a highly-responsive positive feedback loop, independent of biorientation or Aurora-B, a classical regulator of attachment stability. Abrogating Astrin:PP1-delivery disrupts attachment stability and anaphase onset; these can be reversed by artificially tethering PP1 near the C-terminus of Ndc80. Premature constitutive Astrin:PP1-delivery disrupts chromosome congression and segregation, revealing a dynamic safety-lock mechanism that stabilises attachments. Thus, Astrin:PP1 selectively and rapidly stabilises end-on attachments, independent of biorientation, to ensure normal chromosome segregation.

P1989/B232

Modeling Chromosome Attachment, Oscillation and Faithful Segregation during Fission Yeast Mitosis.

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We previously described a minimal mathematical model of fission yeast chromosome segregation based on the stochastic attachment and detachment of kinetochore microtubules. This force-balance model accurately reproduced chromosome alignment, bi-orientation and segregation and predicts chromosome attachment defects such as merotelic attachment when an " aurora B-like " activity is perturbed (*1*, *2*, *3*). We recently implemented microtubule dynamics-dependent features, such as chromosome capture and oscillations to our model. Indeed, during metaphase, the attached sister

chromatids oscillate along the spindle axis. The precise mechanics of this conserved process is poorly understood although it can reveal the dynamics of microtubule-kinetochore attachment. Ndc80p, with its long coiled-coil domain, was previously suggested to connect the microtubules with the kinetochores. Modelling with a minimum number of actors suggests that alternating microtubule poly- and depolymerization without kinetochore detachment, together with an asymmetric generated pulling/pushing force or load-dependent switch between these two states, account for the oscillations. In particular, directed movements of the paired kinetochores toward one pole appear because the states of microtubules coordinate (one side growing and the other shrinking), thanks to the force dependence of the state switching rate. Finally, porting this model to the agent-based cytoskeleton simulator Cytosim paved the way for investigating how these oscillations interplay with other aspects of spindle mechanics. 1. G. Gay, T. Courtheoux, C. Reyes, S. Tournier, Y. Gachet, *J Cell Biol*. **196**, 757-774 (2012). 2. T. Courtheoux, G. Gay, Y. Gachet, S. Tournier, *J Cell Biol*. **187**, 399-412 (2009). 3. H. Mary *et al.*, *J Cell Sci*. **128**, 3720-3730 (2015).

P1990/B233

Multiple Kinetochore Maps Coordinate for Robust End-on Kinetochore-Microtubule Attachments during Mitotic Metaphase.

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Cdt1, a DNA replication licensing protein, is recruited to kinetochores during mitosis via the loop domain of the Ndc80 complex (*Varma et al., Nat Cell Biol., 2012*). Our recent study had demonstrated a direct binding of Cdt1 to microtubules (MTs) and a role for Aurora B kinase phosphorylation in modulating the strength of this interaction (*Agarwal et al., J Cell Biol., 2018*). Besides Cdt1, the Ndc80 loop domain has been shown to recruit another important microtubule-associated protein (MAP), the Ska complex to kinetochores. In this study, we demonstrate an interaction between these two loop-localized MAPs, i.e. Cdt1 and the Ska complex, using multiple biochemical approaches. This interaction was also mapped in mitotic cellular extracts and was found to be essential for proper recruitment of Cdt1 to kinetochores. Total internal reflection fluorescence microscopy (TIR-FM) experiments demonstrated that Ska strikingly augments MT-binding of Cdt1. These findings were substantiated by studies in HeLa cells, where Ska facilitated enhanced localization of Cdt1 on spindle MTs, indicating a synergy between the two MAPs for MT-binding. Consistently, depletion of Ska resulted in reduced binding of Cdt1 to spindle MTs. TIR-FM experiments also show that Cdt1 synergizes with the Ndc80 complex for binding to MTs as proposed by our previous studies (*Varma et al., Nat Cell Biol., 2012*). Our data points to a model where the Ska complex binds to Cdt1 and facilitates its docking on to the Ndc80 loop domain producing a trimolecular complex. We further used computational modelling to understand the interplay between Ndc80, Ska and Cdt1 for the formation of end-on kMT attachments in metaphase. These simulations suggest that the displacement of a possible complex formed by Ndc80-Ska-Cdt1 is significantly higher than that formed by Ndc80 alone or Ndc80-Ska. Further, we find that the rupture force and time of attachment of the kMT interface in metaphase formed by the Ndc80 complex increases in the presence of Ska and/or Cdt1. We postulate that the trimolecular complex formation between Ndc80, Ska and Cdt1 may be necessary to generate robust end-on kMT attachments in *vivo* to cope with the dynamic flux of spindle

MT growth and shrinkage, and contributing to persistent coupling of kinetochores to dynamic MT ends during metaphase and anaphase.

P1991/B234

Quantitative Measurement of Phosphorylation Occupancy Reveals That Both Graded and Switch-like Mechanisms Regulate Kinetochore Functions during Mitosis.

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Faithful chromosome segregation during mitosis requires the formation of bi-oriented attachments between microtubules and kinetochores. The affinity of kinetochores for microtubules is tightly regulated by phosphorylation. Current evidence indicates roles for multiple kinases (Cdk, Aurora, Plk1, etc) and phosphatases (PP1, PP2A, etc) in tuning kinetochore phosphorylation at each stage of mitosis to generate appropriate kinetochore-microtubule (K-Mt) attachment stability to ensure both robust error correction and efficient satisfaction of the SAC. This view assumes that phosphorylation at any particular site is graded through tight coordination between kinases and phosphatases to properly tune K-Mt stability, yet the extent to which kinetochore proteins are phosphorylated (i.e. Absolute phosphorylation occupancy) is unknown. Here, we develop methods using mass spectrometry to measure the absolute phosphorylation occupancy of the Hec1 subunit of the NDC80 complex derived from mitotic cells under different conditions. Multiple Aurora B kinase-dependent phosphorylation sites display variable occupancy ranging from 20%-30% (low nocodazole treatment) to 40%-50% (high nocodazole or STLC treatment). Inhibition of phosphatase activity significantly increases the extent of phosphorylation of these sites indicating a continuous antagonism between kinase and phosphatase activity to establish the absolute occupancy level. In contrast, we identify a new Cdk-dependent phosphorylation site in the same region of Hec1 that switches phospho-occupancy from <20% (high nocodazole treatment) to >90% (low nocodazole or STLC treatment). These data demonstrate that both switch-like and graded mechanisms of phosphorylation regulate an essential protein at the kinetochore-microtubule binding interface to ensure faithful chromosome segregation. We propose a model whereby Cdk1 acts as a switch to convert kinetochores from an “attachment-favorable” state to an “error correction” state which allows Aurora B to act in a graded fashion to finely tune the attachments to achieve proper bi-orientation.

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Measuring Load-bearing Interactions between the Dam1 Complex and Its Multiple Binding Sites in the Ndc80 Complex.

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In mitosis, accurate chromosome segregation requires the kinetochore to stably attach to the dynamic tip of spindle microtubules. The Ndc80 complex is the main microtubule-binding component of the outer kinetochore. Heterodecameric protein complex known as Dam1 enhances the ability for Ndc80 complex to bear load. We previously identified three interaction sites between the Ndc80 and Dam1 complexes. Electron micrographs of Dam1 and Ndc80 complexes binding to microtubules showed that the Ndc80 complex bridges two Dam1 rings in *vitro*. Mutations in any of these three sites disrupt the

ability of the Ndc80 complex to bridge two rings in *vitro and* disrupt proper Dam1 complex localization to the rest of the kinetochore in *vivo*. However, which interaction sites between the Ndc80 and Dam1 complexes play a role in load bearing is still unknown. Ipl1 phosphorylates the Dam1 complex at six different sites: Dam1p S20, S257, S265, S292; Ask1p S200; Spc34p T199. Phosphorylation at Dam1p S20 disrupts oligomerization of the Dam1 complex. Phosphorylation at the other five sites disrupts interaction with the Ndc80 complex. Therefore, I have phosphorylated regions A^{Dam1p}, B^{Ask1p}, or C^{Spc34p} in the Dam1 complex. I utilized the optical trap to measure the strength of the Ndc80 complex attachment to microtubules in the presence of phosphorylated Dam1 complex. Phosphorylation at sites A^{Dam1p} and B^{Ask1p} resulted in partial defects in the ability of the Ndc80 complex to bear load in the presence of the Dam1 complex. Therefore, regions A^{Dam1p} and B^{Ask1p} play a role in establishing load-bearing interactions with the Ndc80 complex on growing microtubule tips, while region C^{Spc34p} does not. To test whether the same regions in the Ndc80 complex play a role in establishing load-bearing interaction with the Dam1 complex, I have generated two lethal insertion mutations along the Ndc80 protein (B^{Ndc80p} and C^{Ndc80p}). The addition of an insertion mutation to either regions B^{Ndc80p} or C^{Ndc80p} did not affect the ability of the Ndc80 complex itself to bind to dynamic microtubules or to bear load. However, region B^{Ndc80p} showed a partial defect in the ability of the Ndc80 complex to bear load in the presence of the Dam1 complex, while region C^{Ndc80p} did not show any defect. In conclusion, two regions (A and B) are required for load-bearing and the function of the third region is being explored.

P1993/B236

Force and Phosphorylation Tune the Performance of the Kinetochore-Microtubule Interface Reconstituted *in Vitro*.

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The dynamic molecular interface between the microtubule (MT) ends and the kinetochore is tightly regulated to ensure proper segregation of mitotic chromosomes to the daughter cells. This regulation is based on phosphorylation of MT-binding domains in the outer kinetochore, as well as the MT-generated force being translated into a biochemical signal by yet unidentified tension sensors. To deconstruct this regulation, we reconstituted the kinetochore-MT interface in *vitro* from purified components. Using Ndc80 and Ska complexes, the two principal MT-binding components of the human kinetochore, we systematically analyzed the contributions of their multivalency, as well as phosphorylation state and presence of the unstructured regions in these complexes, to the coupling efficiency between the reconstituted kinetochore and dynamic MTs. We found that oligomerized Ndc80 complex binds different features of the MTs through different interfaces: the globular calponin homology domain interacts with the stable lattice of the MT, while the unstructured N-terminal tail is required for interaction with the shortening MT end. This was especially important under MT-generated force: force-dependent MT rescue required the presence of dephosphorylated Ndc80 tail, which frequently caused stalls lasting for a second or more. The tailless Ndc80 oligomers, or the full-length Ndc80 oligomers treated with Aurora B stalled MT shortening for a shorter time and failed to rescue MT shortening. Finally, we reconstituted in *vitro* the direct binding between full length Ska and Ndc80 complexes and found that the joint complex was stable towards the active Aurora B kinase and required Cdk1-phosphorylated Ska3 T358/360 and Ndc80 coiled coil. Under the MT-generated force, the presence of Ska increased the duration of Ndc80-dependent stalls whether the Ndc80 tail was absent, present, or phosphorylated, thus promoting MT rescue and opposing the destabilizing effect of Aurora B

phosphorylation. In summary, we conclude that the ability of oligomeric Ndc80 to stall and rescue MT shortening depends on the Ndc80 N-terminal tail and is regulated by Aurora B kinase, while Ska provides an orthogonal pathway to stabilize NDC80-microtubule attachment independently of Ndc80's N-terminus.

P1994/B237

Asymmetric Microtubule Binding-pausing Behavior of Yeast Ring-forming Dam1 Complex Revealed by Ultrafast Force-clamp.

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In dividing yeast cells, spindle microtubules attach to kinetochores with the help of the heterodecameric Dam1 complex, which assembles into the microtubule-encircling rings. When a kinetochore-bound microtubule shortens, it pulls the ring-coupled kinetochore toward the associated spindle pole, whereas the Dam1 ring coupled to its sister kinetochore is dragged toward the microtubule plus-end. How Dam1 complex translocates under pulling force in different microtubule directions has never been examined. To bridge this gap we applied a highly sensitive ultrafast force-clamp to the Dam1 heterodecamers immobilized on the surface of the bead and interacting with the wall of taxol-stabilized microtubule. In this assay, microtubule-wall gliding of Dam1 complex is examined under controlled forces ranging from 3 to 8 pN, imitating the forces and motions experienced by Dam1 when it couples chromosomes to dynamic microtubule ends. Surprisingly, single Dam1 heterodecamers exhibited brief stationary bindings to the microtubule. The frequency and duration of these bindings were increased when Dam1 was translocating toward the microtubule plus-end. Moreover, this asymmetry increased with increasing pulling force, implying a catch-bond-associated translocation mechanism. These results suggest that conformation of the Dam1 ring changes when the ring slides toward or away from the spindle pole. The increased molecular friction of the ring dragged toward the microtubule plus-end could help to explain how chromosome stays attached to the microtubule end while moving under load.

P1995/B238

Individual Mammalian Kinetochores Count Attached Microtubules in a Switch-like, Highly Sensitive Manner to Control Cell Cycle Progression.

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The Spindle Assembly Checkpoint (SAC) prevents anaphase until all kinetochores attach to the spindle. Each mammalian kinetochore binds many microtubules, but how many attached microtubules are required to turn off the checkpoint, and how the kinetochore monitors microtubule numbers, are not known - and central to understanding SAC mechanisms and function. To address these questions, here we systematically tune and fix the fraction of Hec1 molecules capable of microtubule binding. We show that Hec1 molecules independently bind microtubules within single kinetochores, but that the kinetochore does not independently process attachment information from different molecules. Few attached microtubules (20% occupancy) can trigger complete Mad1 loss, and Mad1 loss is slower in this case. Finally, we show using laser ablation that individual kinetochores detect changes in microtubule binding, not in spindle forces that accompany attachment. Thus, the mammalian kinetochore responds specifically to the binding of each microtubule, and counts microtubules as a single unit in a sensitive

and switch-like manner. This may allow kinetochores to rapidly react to early attachments and maintain a robust SAC response despite dynamic microtubule numbers.

P1996/B239

Stress Testing the Kinetochore: Determining the Minimum Number of Ndc80 Molecules for Force Generation and Checkpoint Silencing.

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During mitosis, the kinetochore binds the dynamic plus-ends of up to ~24 spindle microtubules to produce the force needed to move chromosomes and to silence the spindle assembly checkpoint (SAC). The heterotetrameric Ndc80 complex (Ndc80C) plays crucial roles in both these functions. To simultaneously interact with multiple microtubules, the human kinetochore uses ~250 copies of Ndc80C distributed over its ~200 nm diameter disk-like surface. Interestingly, the number of Ndc80C molecules actively engaged with a microtubule continuously fluctuates over the course of mitosis, correlating with changes in the number of kinetochore-bound microtubules (between 12 to 24). Furthermore, recent studies show that kinetochores can segregate the chromosomes accurately when the number of microtubules that they interact with is depressed artificially to 12-14. This observation raises two questions: (1) what is the minimum number of Ndc80C molecules required for proper chromosome segregation and, (2) how does the Ndc80C number affect force generation and SAC silencing? We address these questions by titrating HeLa kinetochores with microtubule-binding mutants of Ndc80C. Specifically, we express fluorophore-tagged Hec1 (a subunit of Ndc80C) with mutations either in its basic N-terminal tail (whose microtubule-binding affinity is biologically modulated by a kinase) or within its calponin homology domain (which has no known regulation on its microtubule-binding affinity). We allow the mutant Hec1 to compete with unlabeled, wild-type Hec1 for kinetochore binding, and then correlate the amount of mutant protein per kinetochore with phenotypes of chromosome segregation. Our initial experiments reveal that the SAC becomes constitutively activated when ≥30 - 40% of the kinetochore is occupied by Hec1 mutants defective in microtubule-binding and silenced when the kinetochore is occupied by ≥60% of a Hec1 mutant with high microtubule affinity. Additionally, we find that chromosome mis-segregation consistently occurs when the kinetochore incorporates ≥20% of high microtubule affinity Hec1 mutant whose attachment strength cannot be modulated. These observations suggest that: 1) SAC silencing occurs when only 60% of the total number of Ndc80C molecules is bound to microtubules and, 2) that proper chromosome segregation relies on the kinetochore's ability to dynamically tune Ndc80C microtubule affinity, failing when as low as 20% of the Ndc80C is in a constitutively high affinity state. Overall, our investigations suggest that the human kinetochore incorporates an excess of Ndc80C and allow us to delineate the limits on this number for proper force generation, SAC signaling, and ultimately, accurate chromosome segregation.

P1997/B240

Proteomic Dissection of the Spindle Assembly Checkpoint.

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Eukaryotic cell division promotes the correct transmission of genetic material from one mother cell to two newly formed daughter cells. To ensure the fidelity of this process, the Spindle Assembly Checkpoint (SAC), a group of proteins that includes Mad1, Mad2, Bub1, BubR1, and Bub3, works in unison to

rigorously verify microtubule-kinetochore attachment and tension in a time-dependent manner. If the SAC senses any errors it halts cell division at the metaphase to anaphase transition until errors are repaired. To gain insight into the associations that are critical for the spatial-temporal regulation of the SAC, we employed a label-free proximity-based association mapping approach. We generated BioID2-tagged inducible stable cell lines for core SAC components (Mad1, Mad2, Bub1, BubR1, and Bub 3) and a control BioID2-tag only cell line. We utilized these BioID2-SAC protein cell lines to perform proximity-based biotin labeling reactions, purified the biotinylated proteins, and analyzed the identity of these proteins by liquid chromatography tandem mass spectrometry. We further developed a statistical proteomic data analysis pipeline called CANVS in R that 1) is user friendly 2) can be used analyze data with an input control and can generate statistically significant results 3) uses the existing CRAPome database 4) generates visual protein association maps from the identified proteins using Reactome. Using these proteomic and computational approaches, we identified known and novel associations for the core SAC proteins, which will allow us to gain insight into the establishment and regulation of the SAC. Interestingly, we found associations between some core SAC proteins and the Astrin/Kinastrin complex. Additionally, we were able to recapitulate the Mad1-Mad2, Mis12-centromere, mitotic checkpoint, and Bub1-Bub3 complexes. Our current and future work is focused on better understanding the significance of these identified associations by analyzing their importance for SAC function.

P1998/B241

Polo Regulates the RZZ-Spindly Module to Ensure Kinetochore-microtubule Attachment Fidelity.

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Accuracy of chromosome segregation relies on the formation and stabilization of correct attachments between kinetochores and microtubules. Ultimately, amphitelic attachments, in which sister chromatids bind to microtubules from opposite spindle poles, must be preserved. Multiple roles have been attributed to Polo kinase in the regulation of kinetochore-microtubule (KT-MT) interactions, promoting either stabilization or destabilization of attachments. However, a more detailed analysis that helps to integrate these functions is still missing. Here, resorting to an RNAi-screen in *Drosophila* to suppress a constitutively active Polo mutant, we identified a genetic interaction between Polo and the Rod-ZW10-Zwilch (RZZ) complex. Expression of constitutively active Polo both in *Drosophila* S2 cells and larval neuroblasts results in delayed formation of stable KT-MT attachments. Importantly, depletion of Rod rescues this delay, confirming a recently reported function for the RZZ complex in the regulation of KT-MT interactions. Mechanistically, Polo phosphorylates Spindly, a kinetochore adaptor for cytoplasmic dynein, impairing its binding to Zwilch. This prevents premature stripping of the RZZ complex from kinetochores and minimizes the stabilization of erroneous attachments that form during early mitotic stages. In agreement with this, expression of different phosphomutant Spindly constructs that affect RZZ kinetochore accumulation results in increased formation of KT-MT attachment defects. We propose a model in which Polo kinase-mediated regulation of the RZZ-Spindly-dynein module during mitosis contributes to ensure the fidelity of KT-MT attachments and chromosome segregation.

P1999/B242

Bub1 Activates the Mad1-mad2 Complex at Unattached Kinetochores to Kick-start Spindle Checkpoint Signaling.

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The spindle checkpoint senses the attachment status of kinetochores, the structures that mediate the interaction between mitotic chromosomes and spindle microtubules, to promote accurate chromosome segregation. When kinetochores are unattached, they recruit the Mad1-Mad2 complex that in turn catalyzes the formation of an anaphase onset inhibitor. How Mad1-Mad2 localization to unattached kinetochores triggers spindle checkpoint signaling is currently unclear. In yeast and human cells, Mad1-Mad2 localization involves a conserved motif (CM) in the Bub1 kinetochore scaffold, which when phosphorylated by mitotic kinases, interacts with the Mad1 C-terminus. However, we found that in *C. elegans*, the Bub1 CM and the Mad1 C-terminus are dispensable for Mad1-Mad2 kinetochore localization, consistent with our previous finding that in this species a different region of Bub1 interacts with Mad1 (Moyle et al. J Cell Biol 204(5):647-57). Nonetheless, we observed that both the Bub1 CM and the Mad1 C-terminus were essential for checkpoint signaling in *C. elegans*, indicating that the interaction between them is critical for activating signaling beyond kinetochore localization; a similar conclusion was derived in yeast and human cell studies using artificial kinetochore tethering of Mad1-Mad2 (Heinrich et al. EMBO Rep 15(3):291-8; Kruse et al., EMBO Rep 15(3):282-90; Ballister et al. J Cell Biol 204(6):901-8). To address how the Bub1 CM interaction with the Mad1 C-terminus activates checkpoint signaling, we focused on the key initiating event of the signaling mechanism: recruitment by kinetochore-localized Mad1-Mad2 of a dynamic, free pool of Mad2 through Mad2-Mad2 dimerization. Employing a fluorescent version of *C. elegans* Mad2 that exclusively localized to kinetochores through dimerization with endogenous Mad2, we found that the Bub1 CM and the Mad1 C-terminus are essential to recruit the dynamic pool of Mad2 to unattached kinetochores. We further show that both the folded RWD domain and putative C-terminal phosphorylation sites in Mad1 are important for this function. These findings lead us to propose that the conserved interaction between phosphorylated Bub1 CM and the Mad1 C-terminus activates the Mad1-Mad2 complex to recruit the dynamic pool of Mad2, thereby triggering checkpoint signaling. Such a mechanism explains coupling of the activation of spindle checkpoint signaling to kinetochore localization of Mad1-Mad2, irrespective of the precise molecular basis for Mad1-Mad2 kinetochore localization. Our current work is testing different models for the activation mechanism, facilitated by the natural dispensability in *C. elegans* of the Bub1 CM and Mad1 C-terminus for Mad1-Mad2 kinetochore localization.

P2000/B243

***Drosophila* KNL1 Regulates the Spindle Assembly Checkpoint through Tension Sensing.**

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The kinetochore protein KNL1 is a large intrinsically disordered protein (IDP) that recruits spindle assembly checkpoint (SAC) proteins and is required for SAC signaling in a conserved manner. Chromosome biorientation satisfies the SAC and while it has been proposed that SAC satisfaction may require the establishment of stable kinetochore-MT (KT-MT) attachments and tension generation, the question of whether tension directly regulates SAC signaling is unresolved and controversial. Here we present data in support of KNL1 as a tension-sensing IDP that directly regulates checkpoint protein localization and signaling independent of the establishment of stable KT-MT attachments. The N-

terminus of *Drosophila melanogaster (Dm)* KNL1 utilizes two basic patches to bind directly to MTs via electrostatic interactions. Pull-down experiments with purified components demonstrated that the checkpoint protein Bub3 binds to the central disordered region of *Dm*KNL1 in the absence of phospho-regulation due to the presence of divergent, “phospho-mimetic” Bub3-interacting motifs in *Dm*KNL1. Deletion of the N-terminal MT binding region (KNL1ΔN) resulted in a 104 +/- 15 minute delay in metaphase compared to an 8.7 +/- 0.8 minute metaphase duration in control cells. KNL1ΔN-expressing cells retained ~2-fold higher levels of Bub3 at bioriented KTs that were hyper-stretched relative to controls. KNL1ΔN-expressing cells established KT-MT attachments that were more stable, as measured by PA-GFP-α-tubulin turnover, than KT-MT attachments in control cells. The delay was fully rescued upon introduction of both the MT-binding protein Tau and a protein phosphatase 1 (PP1) binding motif to the N-terminus of KNL1ΔN. Insertion of a FRET-based tension sensor (TSMod) into its central disordered region revealed that *Dm*KNL1 is a force-transducing KT protein. Single molecule experiments are ongoing to determine if physiological force application to *Dm*KNL1 reduces its affinity for Bub3. We propose that KNL1 acts as a tension sensor to directly regulate SAC signaling independent of KT-MT attachment stability. More specifically, we posit that the central IDP domain of *Dm*KNL1 binds SAC proteins under low tension, and when its N-terminus associates with dynamic MTs, tension-generation reduces its affinity for checkpoint proteins. We see no reason why this mechanical mechanism would not be conserved beyond *Drosophila* in systems where the focus has been exclusively on the phospho-regulation of KNL1.

P2001/B244

The Number and Effectiveness of the Melt Motifs in Spc105 Represents a Compromise for Potent Mitotic Checkpoint Signaling and Its Effective Silencing.

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Maintenance of genome stability and timely cell division are both essential for cell viability and proliferation. To achieve these objectives, a dividing cell must implement a strong mitotic checkpoint that prevents cell division from proceeding in the presence of one or more unattached chromosomes, and but also a responsive one that allows chromosome segregation to occur soon after the last chromosome achieves bipolar attachment with the spindle apparatus. Here we show that the tuning of both the number and the Bub3-Bub1 binding affinity of the ‘MELT’ motifs in the kinetochore protein Spc105 is necessary to implement a potent mitotic checkpoint that can be effectively silenced. Many, high affinity MELT motifs per Spc105 molecule are desirable to prevent chromosome missegregation especially under dynamic signaling conditions wherein the number of unattached chromosomes is small and changing (e.g. In media containing low doses of the microtubule poison benomyl). However, too many high affinity MELT motifs per Spc105 become problematic for checkpoint silencing, and they can result a prolonged cell cycle. Furthermore, high affinity MELT motifs require the binding of Protein Phosphatase 1 to Spc105 for SAC silencing. By recruiting of PP1 via Spc105 specifically for SAC silencing budding yeast cells incur an inherent cost in the form of the harmful cross-talk between SAC silencing and error correction. Therefore, the cumulative action of a combination of high and low affinity MELT motifs represents a compromised solution that achieves a strong and responsive checkpoint. Our findings reveal a potential driver behind the evolutionary trend of the amplification of MELT motif number per Spc105, but the absence of a strong positive selection for their amino acid sequence. We use these insights to engineer an optimal variant of Spc105 that exhibits robust SAC signaling under dynamic signaling conditions, rapid SAC silencing, and improved chromosome biorientation.

P2002/B245

PP1 and PP2A-B56 Remove Kinetochore-localised PLK1 Activity to Silence the Spindle Assembly Checkpoint.

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The phosphatases PP1 and PP2A-B56 localise to the outer kinetochore to silence the spindle assembly checkpoint (SAC) signal. This is thought to occur through dephosphorylation of MELT repeats on the SAC scaffold KNL1, which are otherwise phosphorylated by MPS1 to recruit the SAC machinery to kinetochores. We show here that, surprisingly, these phosphatases are not required to dephosphorylate the MELT repeats directly. Instead, they remove PLK1 from the BUB complex, which can otherwise maintain MELT phosphorylation in the absence of MPS1 activity. This occurs due to regulation of two phospho-binding epitopes on the BUB complex (BUB1-T609 and BUBR1-T620), which are phosphorylated by CDK1 to recruit PLK1. Removing either phosphatase increases phosphorylation at these sites and enhances kinetochore PLK1 levels in wild-type, but not BUB1-T609A/BUBR1-T620A mutant cells. This localised PLK1 prevents MELT dephosphorylation upon MPS1 inhibition because co-inhibition of PLK1, or mutation of its binding epitopes on the BUB complex, can fully restore MELT dephosphorylation and SAC silencing in the absence of either phosphatase. In fact, if PLK1 is inhibited we can find no requirement for PP1 or PP2A in SAC silencing at all, because MELT dephosphorylation kinetics are identical even after strong inhibition of both phosphatases. In summary, this study clarifies how MPS1 collaborates with PLK1 to activate the SAC: MPS1 initiates MELT phosphorylation to recruit the BUB complex and this primes local PLK1 activity to maintain MELT phosphorylation. Kinetochore phosphatases must then ultimately co-operate to remove this co-localised PLK1 and silence the checkpoint.

P2003/B246

Defining the Mechanism by Which Aurora B Contributes to Mitotic Checkpoint Signaling.

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The mitotic checkpoint, also known as the Spindle Assembly Checkpoint (SAC), ensures genomic stability by preventing chromosome missegregation during cell division. It is activated by a conserved mitotic kinase known as Mps1. Mps1 phosphorylates conserved motifs known as ‘MELT motifs’ in the kinetochore protein Spc105/KNL1 specifically within unattached kinetochores. This enables Spc105/KNL1 to recruit SAC signaling proteins, and ultimately results in the production of the Mitotic Checkpoint Complex (MCC) to inhibit anaphase onset. It has long been known that the Aurora B kinase, which is responsible for the correction of erroneous kinetochore-microtubule attachments, also contributes to SAC signaling. However, the underlying molecular mechanism has remained poorly understood. Using an ectopic SAC activation system, we show while Aurora B by itself cannot activate the SAC signaling cascade by phosphorylating the MELT motifs. However, it contributes to MCC production by phosphorylating SAC signaling proteins that the MELT motifs recruit. We also find that unlike Mps1, this contribution of Aurora B to SAC signaling is dependent on the SAC signaling protein Bub1. Finally, the Bub1-dependent Aurora B activity is conserved in both budding yeast and human cells. In summary, Mps1 activates the SAC by phosphorylating the MELT motifs as well as the SAC signaling proteins that they recruit. Aurora B then acts downstream from Mps1 on a subset of the SAC signaling proteins to positively and directly contribute to MCC generation. These findings explain prior

observations regarding the cooperation between Aurora B and Bub1 in SAC signaling. They also suggest an integrative model for the role of kinetochore-microtubule attachment and centromeric tension in activating the mitotic checkpoint.

P2004/B247

Phosphorylation Cascade Regulates Kinetochore Localization of Spindle Assembly Checkpoint Proteins during an aphase.

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The spindle assembly checkpoint (SAC) prevents anaphase onset in response to chromosome attachment defects, and must be silenced for anaphase progression. Following anaphase onset, activated Cdc14 phosphatase dephosphorylates CDK substrates to further facilitate correct chromosome segregation. In budding yeast, Cdc14 dephosphorylates Fin1, a regulatory subunit of protein phosphatase 1 (PP1), to enable kinetochore localization of Fin1-PP1. We previously showed that kinetochore-localized Fin1-PP1 inhibits kinetochore association of SAC protein Bub1. We report here that Fin1-PP1 inhibits this localization by reversing Ipl1 kinase-dependent phosphorylation of the kinetochore protein Ndc80. Therefore, Ndc80 dephosphorylation promotes kinetochore dis-association of SAC protein Bub1 during anaphase. Additionally, phospho-deficient *ndc80* mutants exhibit compromised checkpoint activity in response to tensionless attachments. These results suggest that the removal of SAC protein Bub1 from the kinetochore is regulated by increasing phosphatase activity during anaphase.

P2005/B248

Regulation of Protein Kinase a (pka) Activity to Mediate Chromosome Segregation in Saccharomyces Cerevisiae.

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Chromosome segregation results in the partitioning of sister chromosomes into two new cells with an equal number of chromosomes. This process occurs during mitosis and meiosis and errors can lead to aneuploidy, a karyotypic state in which cells carry the incorrect number of chromosomes. Most of the intrinsic factors that regulate the chromosome segregation process such as the multi-subunit kinetochore complex that connects chromosomes to the mitotic spindle are highly studied and virtually all of the protein subunits have been identified in yeast, flies, worms, and humans. However, much less is known about how extrinsic factors, which include nutrients, temperature, and pH might act on the intrinsic chromosome segregation machinery. Recently, the major glucose signaling pathway (Ras/Protein Kinase A) was shown by our lab to phosphorylate the outer kinetochore subunit, Dam1, and contributes to chromosome segregation fidelity in the budding yeast, *S. cerevisiae*. While this is the first description of a molecular pathway connecting glucose to chromosome segregation many questions remain. In particular, what is the mechanism regulating PKA-mediated phosphorylation of the kinetochore. Is the known Ras/PKA pathway that responds to glucose also directing PKA toward the kinetochore or are there additional signaling components that are specific to PKA's role in kinetochore phosphorylation. As a first step toward determining the mechanism of PKA regulation, we measured the expression and activity of PKA at different cell cycle stages. Our initial results show that PKA activity increases through the cell cycle although protein levels remain constant. Associated with the increase in PKA activity we also observe increasing levels of Dam1 phosphorylated at the PKA site. Our next step is

to determine if the known Ras/PKA pathway components are required for the cell cycle regulation of PKA activity and Dam1 phosphorylation. These studies will bring new insights into how extrinsic factors such as glucose can directly modulate kinetochore function and chromosome segregation fidelity.

P2006/B249

Investigating the Role of KIF18A in Satisfying the Spindle Assembly Checkpoint.

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Progression from metaphase to anaphase during mitosis requires end-on attachments between spindle microtubules and kinetochores. These attachments mature as chromosomes are aligned at the metaphase plate. However, the mechanisms that couple chromosome alignment and attachment are not fully understood. The kinesin family protein, KIF18A, suppresses kinetochore microtubule dynamics to promote chromosome alignment. Interestingly, KIF18A is also necessary, in certain cell types, to satisfy the spindle-assembly checkpoint (SAC). Two general models have been proposed to explain KIF18A's role in satisfying the SAC. In one model KIF18A promotes kinetochore microtubule attachments through an unknown mechanism, and in the other KIF18A is necessary to produce inter-kinetochore tension, which may satisfy the SAC. Our work aims to determine how KIF18A contributes to satisfaction of the SAC. In agreement with previous work, we find that loss of KIF18A results in increased localization of the SAC protein MAD1 to a subset of kinetochores. We used live cell imaging to analyze the behavior of MAD1-positive kinetochores during metaphase and found that these kinetochores exhibit "tumbling" motions rather than oscillations, consistent with an attachment defect. As cells progress from prometaphase to metaphase and kinetochore-microtubule attachments mature during mitosis, phosphorylation of Ser44 and Ser55 of Hec1 decrease. Our data indicate that loss of KIF18A increases phosphorylated Hec1 in metaphase-arrested cells to levels normally seen in prometaphase. These results support a model in which KIF18A coordinates chromosome alignment with attachment and SAC satisfaction during metaphase. Current studies are focused on determining how KIF18A promotes HEC1 dephosphorylation and kinetochore-microtubule attachment.

P2007/B250

Exploring the Link between Centromere Stiffness and Mitotic Dynamics.

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Mitosis involves a number of dynamic processes, such as mitotic spindle formation and chromosome congression during prometaphase, maintenance of spindle position and size in metaphase, and chromosome segregation in anaphase. These dynamic processes are orchestrated by a delicate balance of forces produced by various structural components (microtubules, motors, chromosomes, etc.) of the mitotic apparatus, which can produce forces actively and/or can respond to and transmit forces due to their intrinsic mechanical properties. Recent studies have shown that the centromere and the kinetochore display viscoelastic behavior and that this behavior is conserved in distantly related eukaryotes. In the present study, we aimed to investigate whether changes in centromere stiffness, by changing the transduction of forces through the kinetochore to the microtubules of the mitotic spindle, affect the dynamics of specific mitotic events. To this end, we are using small molecule inhibitors that perturb chromosome compaction and we infer changes in centromere stiffness by measuring inter-kinetochore distance in metaphase cells, with an increase in such distance being interpreted as a

decrease in stiffness and a decrease in the distance as reflective of increased stiffness. Our results so far indicate that the time required to achieve bipolar spindle assembly and chromosome alignment to the metaphase plate changes in response to changes in centromere stiffness. Moreover, we find that altering centromere stiffness suppresses chromosome oscillations in metaphase, regardless of how centromere stiffness is altered. This suggests that centromere stiffness may have been fine-tuned over evolutionary times to facilitate timely and faithful mitotic progression.

P2008/B251

Spatial Positioning and Dynamic Behavior of the Spindle and Kinetochore Associated (SKA) Complex.

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Prior to the segregation of sister chromatids in anaphase, all chromosomes are first maneuvered to the spindle equator in a process termed congression. Bi-orientated sister chromatids achieve this through microtubule depolymerisation-coupled pulling from the leading sister kinetochore. During this process, there is evidence of progressive microtubule recruitment and accumulation of the Spindle and Kinetochore associated (Ska) complex. We proposed that this maturation is part of a mechanical microtubule attachment self-check: if the kinetochore does not recruit sufficient Ska complex, or microtubule recruitment is too rapid, the kinetochore will detach. We have now used CRISPR/Cas9 gene editing to add a fluorescent protein tag to the endogenous Ska1 and investigate how Ska binding/unbinding relates to kinetochore mechanics. GFP-Ska1 appears to be largely absent from the spindle with a dominant kinetochore signal. Moreover, we have deployed our recently developed three-color subpixel co-localization (fGPS) methods to map the position and orientation of the Ska complex within kinetochores as they cycle between attached and unattached states. Finally, we have reconstituted Ska complex behavior on dynamic microtubules in vitro and have evidence for different modes of tracking growing and shrinking tips. Here we will present an updated model for Ska function at human kinetochores.

P2009/B252

Investigating the Link between BubR1 and Hec1 Tail Domain Phosphorylation in Human Cells.

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BubR1 is a kinetochore-associated protein with important roles in both spindle assembly checkpoint signaling and kinetochore-microtubule attachment regulation. It has been previously shown that BubR1 becomes essential for the formation of proper kinetochore-microtubule attachments in a subset of glioblastoma cancer cells, while non-transformed cells do not require this function of BubR1 (Ding et al., 2013. Cancer Discov 3, 198-211). As a component of the Mitotic Checkpoint Complex (MCC), which is required to bind and inhibit the an aphase Promoting Complex/Cyclosome (APC/C) in the presence of unattached kinetochores, BubR1's role in the checkpoint is essential in both cancer and non-cancer cells. In a separate function, BubR1 is responsible for the recruitment of the PP2A-B56 phosphatase complex to kinetochores. This complex works to antagonize the kinase Aurora B, which acts to destabilize kinetochore-microtubule attachments early in mitosis by phosphorylating key substrates at the kinetochore. Why this kinetochore-microtubule attachment function of BubR1 appears to be critical in only transformed cells is unknown. In this study, we are working to understand the specific substrates and sites that the BubR1-recruited pool of PP2A-B56 is responsible for dephosphorylating. We have

tested three phosphorylation sites in the Hec1 tail domain, a known Aurora B kinase substrate. We find that depletion of BubR1 results in increased phosphorylation at all three sites in cells in early mitosis. Additionally, we quantified kinetochore-microtubule attachment stability in BubR1-depleted cancer cells and find that the defects in forming stable attachments are rescued by over-expression of a non-phosphorylatable Hec1 mutant. We are currently working to compare kinase and phosphatase activities in transformed and non-transformed cells to determine if an imbalance of these two components involved in kinetochore-microtubule attachment regulation might explain why cancer cells have an added dependency for the BubR1-recruited pool of PP2A-B56.

P2010/B253

Systematic Phosphorylation an alysis of Bubr1 Kinase Signaling in Mitosis.

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Orchestration of mitotic chromosome segregation depends on a large number of protein machines that regulate the major structural, physiochemical and physiological transitions necessary for equal distribution of parent genomes. Recent studies show that mitotic processes are regulated by acetylation-phosphorylation cascades (Bao et al., 2018. *J. Mol Cell Biol.* 10, 18-32; Yang & Yu. 2018. *J Mol Cell Biol.* 10, 89-90). Although many mitotic phosphorylation events have been identified in proteome-scale mass spectrometry studies, information on how these phosphorylation sites are distributed within mitotic protein complexes and which kinases generate these phosphorylation sites is largely lacking. Give the recent identification of BubR1 chemical inhibitor bubristatin (Huang et al., *Cell Res*. 29, 562-578; Limzerwala & van Deursen. 2019. Cell Res. 29, 605-606), we compared phosphorylation sites in mitotic HeLa cells that were or were not treated with the bubristatin, by phosphopeptide enrichment via immobilized metal affinity chromatography. An alysis by quantitative mass spectrometry identified 313 unique mitotic phosphorylation sites on 163 proteins. Of these, 69 proteins contained one or multiple phosphorylation sites whose abundance was decreased by BubR1 inhibition. These include proteins implicated in BubR1-regulated processes such as senescence, spindle assembly checkpoint signaling, and chromosome segregation, but also numerous proteins that were not suspected to be regulated by BubR1 (Song et al., 2018. *J. Mol Cell Biol.*10, 559-572; Li et al., 2018. *J. Mol Cell Biol.*10, 2-17). An alysis of amino acid sequence motifs among phosphorylation sites down-regulated under BubR1 inhibition in this data set identified the BubR1 consensus phosphorylation motif.

P2011/B254

Investigating the Role of Kinetochore Dynein-dynactin in Spindle Assembly Checkpoint Function.

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The kinetochore is a large proteinaceous structure that assembles upon centromeric chromatin during cell division. This complex structure is involved in linking mitotic chromosomes to spindle microtubules, as well as identifying and correcting erroneous kinetochore-microtubule attachments to ensure accurate chromosome segregation. Monitoring of kinetochore-microtubule attachments is carried out by the spindle assembly checkpoint (SAC), a surveillance system that generates a “wait anaphase” signal at unattached kinetochores. During metazoan mitosis, the minus end-directed motor protein cytoplasmic dynein-1 localizes to a domain at the outer kinetochore called the fibrous corona. Upon stable kinetochore-microtubule attachment, it is thought that kinetochore-localized dynein strips SAC components away from the kinetochore, thus silencing the checkpoint and allowing for anaphase

progression. The three-subunit Rod-Zwilch-ZW10 (RZZ) complex recruits the dynein cargo adaptor Spindly to kinetochores; Spindly, in turn, is responsible for recruiting dynein to the kinetochore. It remains unknown how kinetochore dynein is activated to evict checkpoint proteins away from the kinetochore upon stable microtubule attachment. Here, we investigate the role of dynein at the kinetochore, as well as potential regulatory mechanisms governing Spindly, RZZ, and dynein-dynactin activity. By using a cell culture system that prevents dynein recruitment to kinetochores, we have identified a subset of SAC effectors as bona fide dynein cargoes. Additionally, we have demonstrated that the fibrous corona, a meshwork of proteins at the kinetochore that is supported by RZZS (RZZ + Spindly) and disassembled by dynein, likely has a role in amplification of the SAC signal at unattached kinetochores. Our current work is focused on purification of these kinetochore proteins for use in reconstituted motility assays to further elucidate the nature of their function and interaction.

Oncogenes and Tumor Suppressors 3

P2012/B256

Nuclear Receptor Coactivator SRC-1 Promotes Colorectal Cancer Progression through Enhancing Gli2-mediated Hedgehog Signaling.

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Background and Aims:A growing body of evidence has demonstrated that nuclear coactivators NCOAs (SRCs) are associated with the progression of various cancers and addressing of underlying mechanism represents a promising opportunity to improve cancer therapy. The Hedgehog (Hh) pathway, which is also often aberrantly activated and associated with various tumorigenesis as well. However, the significance of SRC-1 in colorectal cancer (CRC) and the contribution of SRC-1 toward Hh activity have not been characterized yet. **Methods**: SRC-1 knockout CRC cell lines or mouse model was generated to evaluate the role of SRC-1 during CRC tumorigenesis. The SRC-1/GLI2 looping axis was identified through the GSEA analysis of TCGA CRC RNA-seq data and confirmed through RNA and protein expression analysis of clinical CRC samples. The regulation of GLI2 targeted genes by SRC-1 was confirmed through luciferase reporter, Co-IP, and ChIP assays. The cell and xenograft model was used to evaluate genetically or chemically suppression of SRC-1/GLI2 for CRC therapy. **Results**: the ablation of SRC-1 in-vivo significantly suppresses the AOM/DSS induced mouse model of CRC tumorigenesis. Consistently, the mechanistic investigations show that SRC-1 activates the transcription of GLI2, a major downstream transcription factor of Hh pathway and then SRC-1 functions as a coactivator of GLI2 to enhance the expression of multiple GLI2 targeted oncogenes, including GLI2, BCL-2, CCND1, SLUG, and VIM. Furthermore, blockage of SRC-1 and Hh signaling via small molecular inhibitor also largely retard the CRC progression both in human CRC cells and mouse CRC models. **Conclusions**: Together, the present studies uncover an SRC-1/GLI2 regulated Hh signaling looping axis that promotes CRC tumorigenesis, offering an attractive therapeutic strategy for CRC.

P2013/B257

CD82 Regulation of C-met and CD151 in Metastatic Prostate PC3 Cell Lines.

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CD82 regulation of c-Met and CD151 in metastatic prostate PC3 cell lines CD82 (KAI), is a metastasis tumor suppressor protein that is under-expressed in the prostate as well as in several other types of

metastatic cancers. It inhibits cancer metastasis, but the mechanism through which the regulation happens still remains unclear. Various pathways are being explored in our lab, including regulation of c-Met, a growth factor receptor observed from our previous studies that was observed to be regulated by CD82. CD82 and c-Met do not co-localize and recent literature indicates an association exists between c-Met and another tetraspanin, CD151. CD151 does the opposite of what CD82 does ie., it promotes tumor formation. We are currently exploring the link between CD151, CD82, and c-Met. Once we identify what the association of c-Met is with CD151 in the presence and absence of CD82 then it would disclose how exactly the tumor suppressor regulates c-Met and inhibits metastasis. These findings could further help us identify proteins that are regulated by CD82 that could serve as biomarkers or potential drug targets.

P2014/B258

Investigation of PKHD1L1 as a Potential Tumor Suppressor Gene.

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Polycystic Kidney and Hepatic Disease 1-Like 1 (PKHD1L1) was identified in several genome wide association studies as a gene of interest because it is implicated to have a role in cancer development, however little research has been performed to elucidate its function. The Cancer Genome Atlas database search was conducted and missense mutations and variants of PKHD1L1 were found at very high rates across several cancers including skin melanoma, breast and bladder cancer. In addition, these data showed suppressed expression of PKHD1L1 in bladder urothelial carcinoma (Grossman et al., 2016). These clinical data lead us to believe PKHD1L1 could potentially function as a tumor suppressor gene. Besides its association to cancer, PKHD1L1 has been linked to diabetic retinopathy via glucose metabolism experimentation in which hyperglycemic conditions lead to gene suppression (Ung et al., 2017). Immunofluorescent staining revealed that PKHD1L1 appears to be localized to the cytoplasm and nucleus, including nuclear bodies. To characterize PKHD1L1 function, we generated stable knockout lines using CRISPR/Cas9 and observed a frameshift mutation in the targeted exon 2 of PKHD1L1 via TIDE assay. We saw no difference between the parental and knockout cell lines in either wound healing or colony formation assay. However, we observed a modest decrease in proliferation of the PKHD1L1 knockout via MTS assay in reduced glucose culture conditions. These data suggest that PKHD1L1 might trigger a compensatory mechanism in response to reduced glucose levels. Though initial data provide some insight, we require further investigation to help us understand PKHD1L1's role and function.

P2015/B259

Inhibition of Kinase Ikkbeta Suppresses Cellular Abnormalities Induced by the Human Papillomavirus Oncoprotein HPV18E6.

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Human Papillomavirus (HPV) is the leading cause of cervical cancer and has been implicated in several other cancer types including head-and-neck, vaginal, vulvar, penile and oropharyngeal cancers. Despite the recent availability of a vaccine, there are still over 270,000 deaths each year worldwide. Current treatments for HPV-mediated cancers show limited efficacy, and would benefit from improved understanding of disease mechanisms. Recently, we developed a *Drosophila* 'HPV 18 E6' model that

displayed loss of cellular morphology and polarity, junctional disorganization, and degradation of the major E6 target Magi; we further provided evidence that mechanisms underlying HPV E6-induced cellular abnormalities are conserved between humans and flies. Here, we report a functional genetic screen of the *Drosophila* kinome that identified IKK*β*—a regulator of NF-κB—as an enhancer of E6-induced cellular defects. We demonstrate that inhibition of IKK*β* reduces Magi degradation and that this effect correlates with hyperphosphorylation of E6. Further, the reduction in IKK*β* suppressed the cellular transformation caused by the cooperative action of HPVE6 and the oncogenic Ras. Finally, we demonstrate that the interaction between IKK*β* and E6 is conserved in human cells: inhibition of IKK*β* blocked the growth of cervical cancer cells, suggesting that IKK*β* may serve as a novel therapeutic target for HPV-mediated cancers.

P2016/B260

PA28γ Expression Does Not Determine ERα Expression in Mammary Cancer Cells.

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The proteasome activator PA28γ shows increased expression in cancer cells and is positively correlated with cancer severity. Through its role as a proteasome activator, PA28γ has been associated with protein stability and gene expression affecting cancer growth. One possible mechanism in which PA28γ has been connected to breast cancer growth is through regulating levels of the estrogen receptor, ERα. ERα expression can be correlated with the growth and severity of human breast cancers. Many human breast cancers are ERα positive and show estrogen dependent growth. The purpose of this research was to further evaluate whether PA28γ and ERα are correlated across multiple mammary cancer cell types. Furthermore, the gene activity of ERα can be measured by evaluating the transcription rates of ERα gene targets. Cathepsin D, the gene target being measured in these experiments, is a lysosomal protease whose transcription is promoted by ERα. Western blot data showed that cells with increased concentration of PA28γ protein were not significantly correlated to protein concentration of ERα as expected. RT-qPCR data showed that there was no universal correlation between PA28γ concentration and ERα gene expression, which was consistent with the western blot results. Additionally, RT-qPCR demonstrated that Cathepsin D gene expression is not tightly correlated with ERα gene expression. Therefore, alterations in PA28γ expression do not affect ERα expression or activity in mammary cancer cell lines.

P2017/B261

Identification and Functional Characterization of Prooncogenic Regulators and Effectors of the Ser Thr Kinase Map4k4 in Medulloblastoma.

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Migration and invasion of Medulloblastoma (MB) tumor cells cause metastatic disease that is associated with poor prognosis and high morbidity. Blocking tumor cell migration and local infiltration could restrict tumor progression and metastasis in MB and other pediatric malignant CNS tumors. However, molecular mechanisms leading to aberrant migration and tissue invasion in pediatric brain tumors are poorly understood and no targeted anti-metastatic therapies exist till date. We found that the Ser/Thr protein kinase MAP4K4 is upregulated in primary pediatric brain tumors compared to normal cerebellum and demonstrated that it is required for growth factor-induced migration and tissue invasion in MB. MAP4K4

modulates cortical F-actin cytoskeleton and membrane dynamics, which promote invasive cell protrusions at the leading edge of MB tumor cells. However, the molecular mechanisms of MAP4K4 control of cell invasion are still insufficiently understood for the design of novel targeting strategies to prevent MAP4K4-driven tissue invasion. Our objectives are to determine how MAP4K4 controls invasive protrusions and tissue infiltration and to design means to target the underlying molecular mechanisms. To identify interactors of MAP4K4 in MB, we employed a proximity-dependent biotinylation (BioID) approach using MAP4K4 N- or C-terminally fused with a biotin ligase. Streptavidin pull-down followed by mass-spectrometry analysis identified a number of novel putative MAP4K4 effector proteins with known functions in the regulation of cell adhesion and migration and validated their direct or complex-dependent interaction with MAP4K4 through immuno-precipitation. We combined this comprehensive interactor screen with a loss of function phospho-antibody-array analysis, which allowed us to detect activity changes in key signaling pathways that are the result of altered MAP4K4 expression. This approach not only confirmed MAP4K4 implication in pathways controlling cytoskeleton and membrane dynamics but also revealed a previously unknown possible role of MAP4K4 in anti-apoptotic signaling in MB cells. Our data indicate that MAP4K4 contribution to pro-invasive tumor cell functions in MB depends on its presence in supramolecular protein complexes controlling cytoskeleton dynamics and phospho-pathway regulation. We will discuss the potential significance of the proteins identified in the context of invasion control and MB tumor progression.

P2018/B262

Uncovering the Role of a Myosin Phosphatase Regulator in Pancreatic Tumor Cell Mechanics and Behavior.

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Pancreatic ductal adenocarcinoma (PDAC) remains one of the deadliest cancers due to its 5-year survival rate of about 8.5%. A majority of patients present with symptoms post metastatic spread, which contributes to its overall lethality as the 3rd leading cause of cancer-related deaths. There is a critical need for better understanding the molecular mechanisms underlying PDAC formation and progression, which will allow the development of new PDAC-specific targets. To this end, we are investigating the role of the myosin phosphatase targeting subunit 1 (MYPT1) in PDAC as a potential biomarker and lead into the targetability of pancreatic cancer cell mechanics. MYPT1 was originally identified through its being targeted by the immune system in a clinical trial for a cytokine-secreting whole tumor cell vaccine. An antibody response against MYPT1 in patients treated with the tumor cell vaccine correlated to a positive treatment outcome of greater than 3-years of disease-free survival. In addition to MYPT1's elicited antibody response, its expression is highly upregulated in PDAC cells as observed by immunohistological staining of surgically resected patient samples and in established PDAC cell lines. MYPT1 was discovered as an interaction platform between the PP1β/δ catalytic phosphatase subunit and the phosphorylated regulatory light chain (RLC) of non-muscle myosin II (NMII), allowing for RLC dephosphorylation and NMII inactivation. As a major regulator of NMII, MYPT1 has implications in cancer cell shape control, especially during cell growth, division, adhesion, invasion, and metastasis. To elucidate MYPT1's function in PDAC, I am using genetic engineering to generate PDAC cells with varied MYPT1 expression, mimicking the wide range of MYPT1 expression observed in healthy to cancerous states. I am assessing MYPT1's effect on cell mechanics (*e.g*. cortical tension), if MYPT1 responds to applied mechanical stresses, and how MYPT1 affects each of the NMII paralogs' ability to respond to these stresses. I am also characterizing MYPT1's impact on cell oncogenic behavior, including cell

growth, survival, migratory, and invasive phenotypes. Finally, I will characterize MYPT1's effect on tumor formation and progression in *vivo*. Overall, this research will provide insight into MYPT1's biomechanical role in cancer cell behavior and response to its surrounding environment. We aim to provide insight for investigations into MYPT1's targetability and the effect of biomechanical properties on cell oncogenic behavior, which may facilitate its use as a biomarker for precision medicine, leading to a decline in PDAC morbidities.

P2019/B263

Keratin 19 Regulates Cell Cycle Pathway to Promote Proliferation of Breast Cancer Cells.

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Keratin 19 (K19) is overexpressed in select human carcinomas, where it is utilized as a diagnostic and prognostic marker. Despite the positive correlation between higher expression of K19 in tumor and worse patient survival, the role of K19 in breast cancer remains unclear. Therefore, we ablated K19 expression in MCF7 breast cancer cells and found that K19 was required for proper cell cycle progression. Specifically, K19 regulated D-type cyclins (Cyclin D1 and Cyclin D3) expression. We found that K19 interacts with cyclin D3, and a loss of K19 resulted in decreased protein stability of cyclin D3. Since cyclic AMP (cAMP) regulates cell cycle and cyclin D3 degradation, we tested the involvement of K19 in cAMP-mediated cyclin D3 degradation. Elevation of intracellular cAMP through forskolin increased cyclin D3 turnover in the absence of K19. Using this system, we are in the process of characterizing specifics of K19-dependent regulation of cyclin D3 stability. We aim to provide evidences for a novel mechanism by which a cytoskeletal protein stabilizes a cell cycle regulator and promotes proper cell cycle progression.

P2020/B264

The Centrosome Linker Protein, Cep68, Localizes to the Nucleus and Relates to Cell-cell Contact Inhibition.

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CEP68 is an essential structural protein of the centrosome linker, connecting the two centrioles at proximal ends during interphase. When cells enter mitosis, CEP68 is degraded in prometaphase which allows bipolar spindle formation and cell cycle progression. Besides its primary localization and roles at the centrosome, CEP68 can be detected in cell nuclei by immunofluorescence staining. Exogenous expressed N-terminus fragment of CEP68 shows a strong nuclear localization while the full-length protein primarily localizes at the centrosome. Taken together, we hypothesize that the nuclear localization of CEP68 presents a cellular function and is tightly regulated. Interestingly, we discover the nuclear localization of CEP68 is cell density-dependent. CEP68 localizes to the nucleus at low cell density, and exits the nucleus when cultures reach confluence. To elucidate the control of CEP68 localizations, truncated fragments are expressed in cells and examine their localization at different cell density. N-terminus fragments display nuclear localization depending on cell density, while localizations of fragments deleted of this region are not affected by the density of cells. The cell density-dependent nuclear localization of CEP68 is similar to the Yes-associated protein (YAP) of the Hippo/YAP pathway, which regulates the contact inhibition growth. YAP is a transcriptional co-activator and binds to TEAD

transcription factors to promote cell growth. While cells reach confluence, the Hippo kinases (Mst1/2 and Lst1/2) phosphorylate YAP and stimulate its cytosol retention and or degradation. Overexpression of CEP68 causes nuclear retention of YAP as cells reach confluence and affects its phosphorylation state. In addition, CEP68 interacts with upstream Hippo pathway components, Lats and AMOT. Taken together, our results demonstrate that CEP68, an essential structural protein of the centrosome, localizes to the nucleus in response to cell density and is a novel modulator for cell-cell contact inhibition through interacting with the Hippo/YAP pathway.

P2021/B265

***SETD2* Loss Drives Genomic Instability by Increasing CENP-A Levels and Generation of Dicentric Chromosomes.**

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Epigenetic regulation is critical for genome maintenance and identity of the centromere, the site of kinetochore assembly and microtubule attachment during mitosis. Alterations to the “epigenetic code” at the centromere can disrupt the organization and function of the histone H3 variant that specifies the centromere, CENP-A. Changes in CENP-A levels or localization lead to errors in kinetochore assembly and chromosome segregation in mitosis causing aneuploidy, a hallmark of cancer. However, genetic perturbations in epigenetic regulators that function as tumor suppressors or oncogenes have not yet been strongly linked to changes in centromere identity or CENP-A function/localization. Previously, we have identified that loss or mutation of the tumor suppressor and H3 methyltransferase *SETD2* causes errors in chromosome segregation and aneuploidy. Yet, it is unclear how loss of SETD2-dependent histone methylation (H3K36 tri-methylation, H3K36me3) directly leads to chromosome mis-segregation. Here, we demonstrate that loss of *SETD2* increases total CENP-A protein levels and CENP-A levels at the centromere. This increase in CENP-A is accompanied by an increase in expression of the DAXX/ATRX histone chaperone complex, which has been previously demonstrated to regulate ectopic, non-centromeric CENP-A localization. Importantly, loss of *SETD2* leads to the generation of chromosomes with two CENP-A containing centromeres (dicentric chromosomes), which are due in part to the formation of neocentromeres (centromeres forming at non-centromeric locations). Staining of metaphase spreads from *SETD2* mutant cells demonstrates that many dicentric chromosomes contain the kinetochore protein Hec1/Ndc80 at both centromeres, suggesting that both centromeres are active and may contribute to chromosome segregation errors. We propose that *SETD2*-dependent H3K36me3 regulates centromeric identity and acts as a tumor suppressor by inhibiting ectopic CENP-A deposition and dicentric chromosome formation, thereby promoting genome stability.

P2022/B266

Myt1 Upregulation Protects Cells from Centromere Fragmentation in the Presence of Adavosertib.

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During interphase, Wee1 and Myt1 kinases negatively regulate Cdk1 through phosphorylation. Ectopic activation of Cdk1 can induce mitosis with damaged or under-replicated DNA, which results in cell death by mitotic catastrophe. Adavosertib is a potent small molecule inhibitor of Wee1 that has single agent clinical activity in multiple solid tumours, including sarcoma, glioma, head and neck cancer, and ovarian

cancer. In HeLa and a subset of breast cancer cell lines, Adavosertib treatment forces S-phase cells into mitosis with under-replicated chromosomes resulting in chromosome breaks at the centromere (centromere fragmentation) and mitotic cell death. However, not all cancer cells respond to Adavosertib and the reason for the lack of response is unclear.We find that cells exhibiting intrinsic resistance to Adavosertib have higher Myt1 protein levels relative to those with lower Myt1 levels. Furthermore, cancer cells (HeLa and MDA-MB-231) that are selected for Adavosertib resistance have 2 to 3 times more Myt1 relative to parental cells. Additionally, tumour cells within xenografts that survival Adavosertib treatment also have higher Myt1 protein levels relative to non-treated xenografts from control mice. siRNA knockdown of Myt1 restores cell sensitivity to the Wee1 inhibitor in resistance cells whereas transient overexpression of Myt1 induces Adavosertib resistance in otherwise sensitive cell lines. We confirmed that Adavosertib resistant cells are less prone to undergoing premature mitosis and centromere fragmentation in the presence of the Wee1 inhibitor. Consistent with this finding, we find that cells with high levels of Myt1 (including cells selected for Adavosertib resistance) have low in vitro Cdk1 activity.We compared patient breast tumour tissue and observed that Myt1 is overexpressed relative to normal breast tissue. Furthermore, higher Myt1 levels strongly correlate with a worse overall survival and an increase in disease relapse. Given the correlation between high Myt1 levels and Adavosertib resistance in pre-clinical models, assaying Myt1 levels in future clinical trials may be useful in identifying resistant tumours. Our data also highlight a rationale for developing small molecule Myt1 inhibitors for clinical use.

P2023/B267

Restoring Chemosensitivity in Multiple-Drug Resistant Breast Cancer through Activation of the an aphase Promoting Complex.

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Common malignancies of the breast, colon, and blood are notable for exhibiting high rates of inherent, or more frequently, acquired resistance to therapy. This transformation into multiple drug resistant (MDR) cancer is associated with poorer patient outcomes as this recurrence is both more aggressive and difficult to treat. One mechanism of developing MDR is the aberrant chromosomal segregation occurring during an abnormal mitotic cycle, inducing chromosomal damage. Accumulation of chromosomal damage may result in subpopulations of cancerous cells developing MDR characteristics. The an aphase Promoting Complex (APC) is a multi-subunit E3 ubiquitin ligase which drives regulated chromosomal segregation during mitosis, and whose proper function is necessary to prevent chromosomal damage. The APC functions by polyubiquitination followed by proteasomal degradation of multiple protein targets including securin, whose degradation permits release and segregation of sister chromatids. The APC is highly evolutionarily conserved both structurally and functionally; from yeast to humans. There is substantial evidence for APC dysfunction during tumor development, and our previous work has associated impaired APC activity with MDR cell populations. We hypothesize that restoring APC activity in MDR breast cancer will result in the enhancement of chemosensitivity. A yeast 2-hybrid system identified multiple novel peptides that physically bind and activate the yeast APC, and through evolutionary conservation, are predicted to similarly activate human APC. Multiple peptides were cloned into mammalian expression vector pcDNA3.1 and stably transfected into a triple-negative breast cancer cell line (innately chemoresistant). Peptide-expressing cells revealed increased APC activity, as several APC protein targets had significantly reduced abundance, presumably due to augmented E3 ligase

activity. These same cells had significantly elevated chemosensitivity to doxorubicin, a common breast cancer chemotherapeutic drug. Furthermore, analysis of the marker for DNA damage H2A.X revealed a reduction in protein quantity. This indicates that cancer cells which have enhanced APC activity correlate with reduced rates of chromosomal damage. Whether this is because heavily damaged cells are removed through apoptosis (or other measures) or there is enhanced DNA repair is yet to be determined. This supports our hypothesis that restoring APC activity in MDR cancer results in enhanced chemosensitivity.

P2024/B268

Role of Jumonji-C Histone Demethylase in the Development of Hepatocellular Carcinoma.

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Reprogramming in lipid metabolism has received increasing recognition as a hallmark of cancer cells. In cancer cells, large demands of lipids were required to meet excessive synthesis of membranes, energy production and activation of intracellular signaling pathways during cell proliferation and division. To meet these demands, cancer cells express increased levels of lipogenic enzymes responsible for FAs synthesis, which is a target of crucial transcriptional regulator, sterol regulatory element binding protein 1c (SREBP1c). SREBP1c is a well-known master transcription factor for de novo lipogenesis and is found frequently increased in many human cancers. However, the molecular mechanisms involved in the regulation of SREBP1c remain incompletely understood. In this study, we uncover the role of jumonji-C histone demethylase (JHDM) as a repressor of SREBP1c, thereby preventing lipogenesis and proliferation of hepatocellular carcinoma (HCC) cells. JHDM directly interacts with SREBP1c at basic helix-loop-helix domain of SREBP1c and plant homeodomain of JHDM, promoting proteasomal degradation of SREBP1c protein. In addition, knockdown of JHDM stimulates mRNA levels of lipogenic enzymes and induces lipogenesis in HCC cells. However, JHDM-loss-induced lipogenesis is recovered by SREBP1c knockdown, implying that SREBP1c is crucial for the effects of JHDM toward lipogenesis. In contrast, ectopic expression of JHDM shows protective effects on oleic acid-induced lipid accumulation in HCC cells. We also found that JHDM knockdown using si-RNAs promotes mRNA expressions of cell cycle regulators, accompanied with enhanced cell growth, colony formation and spheroid formation. However, the effects of JHDM knockdown which promote HCC proliferation are reversed by knockdown of SREBP1c. In accordance with these data, JHDM overexpression represses cell growth in HCC cells, indicating that JHDM acts as a tumor suppressor by inhibiting SREBP1c-mediated lipogenesis and cell cycle regulation. Overall, our findings suggest that JHDM serves as a molecular bridge between lipid metabolism and cancer development through regulation of SREBP1c and thus JHDM could be a therapeutic target in defense against HCC.

P2025/B269

Induction of Fabp by Fatty Acid Is Crucial for Switching on Hif-driven Lipid Accumulation and Cell Growth in Hepatocellular Carcinoma.

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As a hallmark of cancer malignancy, reprogramming of lipid metabolism is currently rising since lipid metabolic abnormalities in cancer are distinguished from normal cells. Increasing demands for rapid growth even under harsh cancer microenvironment, cancer cell alters increasing uptake of exogenous fatty acid and accumulation of lipid droplets, and decreasing beta-oxidation. Previous researches

focused on metabolic changes mediated by Hypoxia Inducible Factor-1 (HIF-1) have been discussed intensively, however, its precise mechanism in reprogramming of lipid metabolism is to be fully clarified. Here, we report that a novel HIF-1 binding partner, Fatty acid binding protein (FABP), is required for switching on reprogramming of lipid metabolism through reinforcing HIF-1 function. Mechanistically, we demonstrate that FABP binds to N-terminal of HIF-1 and enhances HIF-1 activity. Furthermore, treatment of fatty acid triggers FABP gene expression and sequentially activates HIF-1 function in hepatocellular carcinoma (HCC) cell. Up-regulated FABP under fatty acid enriched condition results in HIF-1-mediated shift toward lipid storage and accelerates HCC cell growth. We utilize bioinformatics analysis and clinical data to confirm FABP and HIF-1 expression in the liver of HCC tissues, and both expressions are up-regulated in HCC patients and positively correlated. Finally, we demonstrate that Fatty acid/FABP/HIF-1 axis facilitates HCC proliferation using colony formation and three-dimensional culture. Our findings suggest important roles of fatty acid induced FABP/HIF-1 signaling pathway on lipid accumulation and proliferation in HCC, and this axis might be a potential therapeutic target for metabolism related development of hepatocellular carcinoma.

P2026/B270

Heat Shock Factor 1 Is a Direct an tagonist of AMP-activated Protein Kinase to Promote Tumor Growth.

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Rationale: AMP-activated protein kinase (AMPK), a key cellular metabolic sensor and downstream effector of the tumor suppressor LKB1, activates catabolic pathways but suppresses anabolic pathways, including fatty acid and cholesterol biosynthesis, thereby maintaining energy homeostasis. Heat shock factor 1 (HSF1) is the master transcriptional regulator of the evolutionarily conserved proteotoxic stress response, thereby preserving proteomic stability upon environmental insults. HSF1 is also a potent pro-oncogenic factor, suggesting that proteomic stability enables oncogenesis. We previously showed that upon activation by metabolic stressors, AMPK interacts with and phosphorylates HSF1 to inactivate it and cause proteomic instability, leading to tumor suppression. Intriguingly, we also discovered that HSF1 suppresses AMPK and controls body fat mass. **Objective:** to investigate the mechanisms by which HSF1 suppresses AMPK and the roles of this suppression in lipid metabolism and tumorigenesis. **Results:** by utilizing recombinant proteins, HSF1 peptide libraries and transcription-deficient HSF1 mutants, our in *vitro* studies reveal that through physical interactions HSF1 imposes multilayer regulations on AMPK, including blocking AMP binding to γ subunits, impairing LKB1-mediated Thr172 phosphorylation, promoting Thr172 de-phosphorylation by PP2A, and impeding ATP binding to catalytic α subunits. Circular dichroism spectroscopy reveals that HSF1 induces global AMPK conformational changes. Biologically, *Hsf1* deficiency suppresses lipogenesis and decreases lipid content via AMPK activation. Moreover, *Hsf1*-deficient cells and mice display reduced cholesterol levels. Interestingly, this defect leads to impaired cholesteroylation of sonic hedgehog (SHH). Consequently, the oncogenic SHH signaling is impaired in *HSF1*-deficient cells. In *vivo*, *Hsf1* deficiency reduces body fat mass in mice, which can be markedly rescued by either pharmacological or genetic inhibition of AMPK. Importantly, the transcription-deficient HSF1 mutant, through AMPK suppression, enhances the lipid content and SHH cholesteroylation, and promotes the in *vivo* growth of xenografted human melanomas. **Conclusions:** HSF1 is a direct AMPK antagonist. This transcription-independent interaction of HSF1 with AMPK

epitomizes a reciprocal kinase-substrate regulation whereby lipid metabolism and proteomic stability intertwine and promotes cancer anabolism and oncogenesis.

P2027/B271

Increased NAD(H) Pool Promotes Colon Cancer Progression by Suppressing Ros Level.

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Nicotinamide adenine dinucleotide (NAD), a coenzyme with crucial roles in various cellular processes, exists in an oxidized form (NAD^+) and reduced form (NADH). While cancer cells require high rate of NAD^+ turnover than normal cells, it remains unclear whether the NAD(H) pool could be used as biomarker for cancer progression. Here, we showed that NAD(H) pool size (NAD^+ and NADH) and NAD^+/NADH ratio both were increased during colorectal cancer (CRC) progression. The NADH fluorescence intensity measured by two-photon excitation fluorescence (TPEF) microscopy was consistently increased in CRC cell lines, azoxymethane/dextran sodium sulfate (AOM/DSS)-induced CRC tissues and tumor tissues from CRC patients. We also confirmed that the increase of NAD(H) pool was due to the activation of NAD^+ salvage pathway mediated by nicotinamide phosphoribosyltransferase (NAMPT). The NAMPT expression was upregulated in adenoma and adenocarcinoma tissues from CRC patients. The increases in the NAD(H) pool inhibited the accumulation of excessive ROS levels induced by inflammation. FK866 treatment decreased the CRC nodule size by increasing ROS levels in the AOM/DSS mice. Collectively, our results suggest that NAMPT-mediated upregulation of the NAD(H) pool protects cancer cells against detrimental inflammation-induced ROS and detection of NADH fluorescence by TPEF microscopy could be a potential non-invasive method for monitoring CRC progression.

P2028/B272

Ror1 Is a Key Regulator of Chemoresistance in Breast Cancer Via Modulation of the Drug Efflux Pump Abcb1 in a P53/mapk-dependent Manner.

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ROR1 Is a Key Regulator of Chemoresistance in Breast Cancer Via Modulation of the Drug Efflux Pump ABCB1 in a P53/mapk-dependent Manner Resistance to chemotherapy is one of the leading causes of mortality in most human cancers. In most instances, these tumorous masses exhibiting resistance to chemotherapy require higher treatment doses with toxic side effects for patients. In breast cancer especially, subsets of chemoresistant cells often contribute to dismal survival rates. Understanding the molecules and pathways underlying chemoresistance is critical in order to combat these malignancies. To this aim, our work identifies and characterizes one such clinically targetable regulator of chemoresistance. Our focus is the Receptor Orphan tyrosine-kinase-like Receptor 1 (ROR1), an oncofetal receptor highly expressed during development but absent or minimally expressed in normal adult tissue. It is however, highly expressed in chemoresistant cancerous tissue. We previously isolated and characterized a novel ROR1 inhibitor which represses basal-chemoresistant breast cancer survival and motility. Our results also suggest that chemoresistant breast cancer cells and patient tissue express high levels of ROR1. siRNA knockdown and inhibition of ROR1 increased sensitivity to Cisplatin and Doxorubicin, two widely used chemo drugs. We also observed downregulation of ABCB1 expression after ROR1 knockdown and inhibition in these cells. ABCB1 is a drug efflux pump, highly expressed in chemoresistant malignancies, which pumps out chemo drugs from cells. Mechanistically, after ROR1

knockdown and inhibition, we observed downregulation of MAPK/ERK signaling, a pathway known to prevent proteasomal degradation of ABCB1 via RSK1. On the transcriptional level, when p53 is increased at the ABCB1 promoter, ABCB1 transcription is repressed. SMARCB1 was similarly shown to repress ABCB1 transcription. ChIP analysis suggests ROR1 knockdown and inhibition increases p53 and SMARCB1 at the ABCB1 promoter, which correlated with decreased ABCB1 mRNA levels. We also observed a global increase in p53 activity and nuclear localization in both conditions. Overall our findings suggest ROR1 inhibition with drugs like Cirmtuzmab® and strictinin might represent a viable therapeutic strategy for chemoresistant malignancies.

Tumor Invasion and Metastasis 2

P2029/B273

Metabolic Stress Induced Aggression in Non-invasion Breast Tumor Model.

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An actively growing tumor face various alternation in microenvironment during its life cycle. Changes such as hypoxia, scarcity of nutrients and mechanical confinement most often influence the tumor behavior. The metabolic crosstalk between the tumor and the microenvironment might help in shaping the tumor pathophysiology. We hypothesize the tumor as a smart enterprise, which can revise their internal wiring to survive such stress conditions. The main objective of this study is to understand the impact of extrinsic stress conditions on tumor growth and migration. Non-invasive multicellular spheroids (MCF7) are fabricated and subjected to individual or combined starvation of glucose, protein, and oxygen, to create chronic and acute stress conditions. It is observed that combined stress conditions alter the physical appearance of the tumor, while chronic combine stress deters the in *vitro* spheroid formation thus mimicking the invasive one. This is further strengthened by the reduced level of mRNA expression of cell adhesion specific proteins such as E-cad and EpCAM. Migration of the spheroids is analyzed under different conditions. Continually stressed spheroids exhibit dormant behavior and do not migrate while, in rescued condition, spheroids from acute stress showed epithelial migration. Interestingly rescued spheroids from extreme nutritional stress show single-cell migration. Chronically stressed spheroids exhibit epithelial migration too after the rescue. In the acute combined stressed spheroids, both single and collective mode of migration is observed. To analyze the nature of single-cell migration inhibitors of mesenchymal and amoeboid migration have been used. Inhibitors such as ML7 Hydrochloride restrict the single-cell migration, whereas Bisindolylmaleimide-I and Marimastat cannot. Several cellular events such as the formation of apoptotic bodies, cell-in-cell structures, the formation of cytoplasmic extensions are also observed which need further probing to prove their importance in the process of metastasis. In conclusion, we can assume that nutritional and hypoxic stress conditions synergistically influence the metastatic potential of cancer cells and can inculcate a quasi-invasive nature to a non-invasive cell line. This study highlights the potential of such metabolic stress conditions on metastasis which need more attention in the future.

P2030/B274

Metabolic Control of Tumor Cell Invasion by Lipid Droplet Storage and Metabolism.

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Metastatic invasion is a particularly energy-intensive cellular process, and how tumor cells fuel the energetic demands of metastasis remains poorly defined. Obesity is a risk factor for multiple cancer types, including pancreatic cancer (PDAC), and correlates with increased metastasis. Excess lipids, present due to obesity or increased fatty acid synthesis, could provide a fuel source for cancer progression and metastasis. We hypothesized that tumor cells can store and metabolize excess lipid to drive metastatic invasion. Indeed, introduction of excess exogenous fatty acids augments lipid accumulation and storage in lipid droplets (LDs) in PDAC cells, and leads to enhanced PDAC cell motility and invasion in *vitro*. LDs are metabolized by the concerted action of lipases. Importantly, we have found that treatment of tumor cells with lipase inhibitors blocks tumor cell invasion, indicating that utilization of stored lipids via lipase-mediated LD breakdown is required for invasive migration. Hormone sensitive lipase (HSL) is a lipolytic enzyme, best characterized in adipose tissue, that is critical to the breakdown and utilization of LDs in *vitro* and in *vivo*. We have made the surprising observation that oncogenic KRas downregulates expression of HSL in pancreatic cancer cells, which shifts the cells towards lipid storage. In PDAC, mutational activation of the oncogene KRas occurs in 95% of tumors and contributes to metabolic rewiring of tumor cells. Our data suggest that the metabolic shift induced by mutant KRas includes increased lipid storage in LDs via downregulation of HSL. Indeed, re-expression of HSL shifts tumor cell metabolism from glycolysis towards elevated oxidative phosphorylation. Importantly, KRas-mutant cells still require the catalytic activity of HSL for tumor cell invasion, even though HSL expression is downregulated. Using redox ratio imaging, we have found that migratory cells upregulate oxidative phosphorylation during the process of migration to metabolize stored lipid droplets and fuel invasive migration. Finally, depletion of LD stores via overexpression of HSL blocks tumor cell invasion in *vitro* and metastasis in mice, even in the context of oncogenic KRas. These data indicate that regulation of HSL is important for both lipid storage and maintaining cancer cell invasiveness. Therefore, we propose a novel mechanism by which oncogenic KRas mediates HSL activity in a tunable manner, thereby transiently altering the metabolism of PDAC cells and enabling increased metastatic ability.

P2031/B275

Elovl2 Promotes Breast Cancer Malignancy by Regulating Fatty Acid Biosynthesis.

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<META NAME="author" CONTENT="하유진"> ELOVL2 promotes breast cancer malignancy by regulating fatty acid biosynthesis *Yu-Jin Ha, Hyun-Jin Noh, Han-Hee Park, Min-Kyoung Kim, Yu-Sang Lee and You-Sun Kim Department of Biochemistry, Ajou University School of Medicine & Department of Biomedical Sciences, Graduate School, Ajou University, Suwon, KOREA* Metabolic rewiring has been recognized as an important feature to the progression of cancer. However, the essential components and functions of lipid metabolic networks in breast cancer progression are not fully understood. In this study, we investigated the roles of altered lipid metabolism in the malignant phenotype of breast cancer. Using a spheroid-induced epithelial-mesenchymal transition (EMT) model, we conducted multi-layered lipidomic and transcriptomic analysis to comprehensively describe the rewiring of the breast cancer lipidome during the malignant transformation. A tremendous homeostatic disturbance of various complex lipid species including ceramide, sphingomyelin, ether-linked phosphatidylcholines, and ether-linked

phosphatidylethanolamine was found in the mesenchymal state of cancer cells. Noticeably, polyunsaturated fatty acids composition in spheroid cells was significantly decreased, accordingly with the gene expression patterns observed in the transcriptomic analysis of associated regulators. For instance, the up-regulation of SCD, ACOX3, and FADS1 and the down-regulation of PTPLB, PECR, and ELOVL2 were found among other lipid metabolic regulators. Significantly, the ratio of C22:6n3 (docosahexaenoic acid, DHA) to C22:5n3 was dramatically reduced in spheroid cells analogously to the down-regulation of ELOVL2. Following mechanistic study confirmed the up-regulation of SCD and down-regulation of PTPLB, PECR, ELOVL2, and ELOVL3 in the spheroid cells. Furthermore, the depletion of ELOVL2 induced metastatic characteristics in breast cancer cells via the SREBPs axis. A subsequent large-scale analysis using 51 breast cancer cell lines demonstrated the reduced expression of ELOVL2 in basal-like phenotypes. Breast cancer patients with low ELOVL2 expression exhibited poor prognoses (HR = 0.76, CI = 0.67–0.86). Collectively, ELOVL2 expression is associated with the malignant phenotypes and appear to be a novel prognostic biomarker in breast cancer. In conclusion, the present study demonstrates that there is a global alteration of the lipid composition during EMT and suggests the down-regulation of ELOVL2 induces lipid metabolism reprogramming in breast cancer and contributes to their malignant phenotypes.

P2032/B276

The Creatine Phosphagen System in Mechanoresponsive in Pancreatic Ductal Adenocarcinoma Cells and Fuels Invasive Behaviour.

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Unbalanced cues from the extracellular matrix (ECM) govern pancreatic tumorigenesis and dissemination. Stiff fibrotic stroma and cancer cell mechanosensing regulate cell proliferation, migration and invasion. Such key processes are highly dependent on energy availability and need to be fueled by metabolic adaptations to tumorigenic microenvironments. However, it is still unknown how ECM mechanics influence cellular energetics and metabolism as well as how cells meet their ATP requirements during invasion and metastasis. We found that pancreatic cancer cells tune their metabolic networks to favour ATP production on stiff ECM. This process appeared dependent on mitochondrial fusion and polarization on stiff substrata as well as in pseudopods formed during ECM invasion. In addition, this was accompanied by a mechano-dependent regulation of ATP recycling through the creatine phosphagen system. The cytoplasmic creatine kinase CKB was expressed on stiff substrata in a YAP-dependent manner providing a critical advantage to the invasion capacity of pancreatic cancer cells. Collectively our results indicate that ECM mechanics can positively regulate tumour invasion by favouring ATP production and sharpening the gradient of ATP vs ADP through CKB activity. Our study highlights the importance of this ATP recycling circuit as a mechanism generating an efficient energy balance within the cytoplasm boosting invasion. Interestingly, targeting creatine phosphorylation hindered migration and invasion in 2D and 3D, revealing CKB as a potentially druggable target against the spread of pancreatic cancer.

P2033/B277

Mitochondrial Localization and Plasticity within MDA-MB-231 Cell Migration in Vitro.

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Triple negative breast cancer cells, MDA-MB-231, employ the Warburg effect during growth. During cellular migration the Warburg effect may not be the main ATP source. To test the hypothesis that mitochondrial ATP is an important source of energy for leading edge dynamics in migrating MDA-MB-231 cells, relative locations and morphology of mitochondria (Mito) were quantified during migration in a scratch wound assay. Confluent cell monolayers were grown in glass bottom dishes, a scratch was made through monolayer, and cells were stained with Mitotracker Green FM and Hoescht 33342 for one hour at 37°C. Cells were treated with increasing concentrations of EGF (epidermal growth factor) [0 (n=97), 10 (n=67), 100ng/ml (n=65)] one hour post staining and imaged using scanning laser confocal microscopy. Max Intensity Projections of Z stack images were analyzed to observe Mito through entirety of cell thickness. Thresholding DAPI and FITC channels identified nuclei and Mito. Determining nuclei centroids and direction of migration, cells were split into anterior and posterior regions of interest (ROI). Mitochondrial Localization Index (MLI) was determined by comparing sum mitochondrial fluorescence in anterior ROI to reciprocal posterior ROI; and MLI's were defined as posterior (0-0.35), peri-nuclear (0.35-0.7) or anterior (0.70-1.0). In the absence of EGF (0ng/ml, n=38) cells migrating into scratch wound had significantly more Mito in the anterior region than non-migratory cells (n=59) ($p=0.004$). Increasing concentrations of EGF however decreased the number of Mito localized in anterior regions significantly (ANOVA, $p=0.049$). This was unexpected as EGF increases rates of migration in scratch wound assays. Lamellipodia localized Mito morphology was compared across EGF treatments. Mito were characterized as fused tubular networks or fissioned. Fissioned Mito predominated in lamellipodia of migrating control and EGF treated cells [0ng/ml= 60.5%, 10ng/ml = 64.18%, 100ng/ml = 61.5%] (Logistic Regression $p<0.0001$). In conclusion, Mito localize in the anterior region during migration. EGF results in a decrease in total anterior Mito, but an increase in relative numbers of fissioned Mito in lamellipodia. These data support the hypothesis that Mito are necessary for leading edge dynamics but does not support EGF inducing anterior localization of Mito.

P2034/B278

Aquaporin-7 Is a Metabolic Sensor That Regulates Response to Cellular Stress in Breast Cancer.

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Misregulation of nutrient signaling contributes to cancer growth and treatment response. Using GC-MS, LC-MS/MS, and capillary zone electrophoresis (CZE)-MS platforms, we quantified and compared the levels of 374 metabolites in breast tumor tissue from normal tissue and transgenic mouse breast cancer models overexpressing a panel of oncogenes (PyMT, PyMT-DB, Wnt1, Neu, and C3-TAg). We then effectively integrated metabolomics and gene expression data from breast cancer mouse models through a novel unbiased correlation-based network analysis and have identified 35 metabolite and 34 gene hubs with the most network correlations. These hubs have prognostic value and are likely integral to tumor metabolism and breast cancer. We focused on the gene hub Aquaporin-7 (AQP7), a water and glycerol channel, as a novel regulator of breast cancer. AQP7 is prognostic of overall survival in breast cancer patients. In mouse breast cancer models, reduced Aqp7 expression caused reduced primary tumor burden and lung metastasis. Metabolomics and complex lipid profiling of cells and tumors with

reduced Aqp7 revealed dramatically altered lipid metabolism, glutathione metabolism, and urea/arginine metabolism compared to controls. These data identify Aqp7 as a critical regulator of the metabolic and signaling responses to environmental cellular stresses in breast cancer, highlighting AQP7 as a potential cancer-specific therapeutic vulnerability. Because breast cancer cells with Aqp7 deficiency are sensitive to these perturbations, Aqp7 is an attractive therapeutic target.

P2035/B279

Confined Cancer Cell Migration Causes DNA Damage by Increasing Replication Stress.

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Cancer metastasis i.e., the spreading of cancer cells from primary tumors to distant sites in the body, is responsible for over 80% of cancer related deaths. During this process, cancer cells often migrate through very small interstitial spaces about 1-2 µm in size, i.e., much smaller than the cell diameter. Deformation of the nucleus, which is the largest and stiffest organelle in the cell, can form a rate-limiting step during such confined 3D migration. As cancer cells squeeze through confined spaces, they incur extensive nuclear deformation, and frequently exhibit nuclear envelope rupture and DNA damage. Here, we investigated the molecular mechanism responsible for the migration-induced DNA damage. We used a panel of different cancer cell lines, custom-built microfluidic devices that mimic the interstitial spaces encountered by cells in *vivo*, and fluorescent reporters to detect nuclear envelope rupture, DNA damage, and cell cycle phase. We found that in some cancer cell lines, DNA damage associated with nuclear envelope rupture occurred in all cell cycle stages, suggesting that influx of cytoplasmic nuclear may be responsible. Intriguingly, in other cell lines, nuclear deformation was sufficient to induce DNA damage, even in the absence of nuclear envelope rupture. Furthermore, DNA damage associated with nuclear deformation occurred predominantly in the S/G2 phase of cell cycle, suggesting that the damage might be associated with replication stress. Supporting this hypothesis, the DNA damage was frequently localized at stalled replication forks inside the confined spaces. Physical compression of cells was sufficient to reproduce the confinement-induced DNA damage, confirming that nuclear deformation can induce replication stress. This study, thus introduces a novel concept wherein physical confinement of the nucleus can lead to increased replication stress and replication fork stalling, providing new insights on possible causes of DNA damage during cancer cell migration.

P2036/B280

Confined Migration Induces Heterochromatin Formation in Cancer Cells.

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During cancer metastasis, cancer cells migrate through confined interstitial spaces, requiring extensive deformation of the cell body and nucleus. The severe physical stress on the nucleus during this frequently results in rupture of the nuclear envelope (NE) and nuclear fragmentation. Previous studies

found that mechanical force application on cells can induce mechanosensitive gene expression and chromatin rearrangement. However, it remains unclear whether physical stress on the nucleus during confined migration can induce histone modification and gene expression changes. Here, we performed live-cell and immunofluorescence imaging of HT1080 fibrosarcoma and MDA-MB-231 breast cancer cells migrating through collagen gel matrices and custom-made microfluidic migration devices that mimic interstitial spaces *in vivo*. Cell migration in confined 3D environments resulted in increased H3K9me3 and H3K27me3 heterochromatin marks and DNA methylation compared to unconfined conditions. The heterochromatin enrichment was particularly prominent in cells with visible nuclear deformation and in chromatin protrusions that formed inside nuclear blebs. In addition, we observed GFP-labeled HP1α enrichment within nuclear blebs in HT1080 and MDA-MB-231 cells during confined migration. By using a fluorescence recovery after photobleaching (FRAP) assay, we verified those GFP-HP1α enrichments to be heterochromatin. Treating cells with a pan histone methyltransferase inhibitor 3-Deazaneplanocin a (DZnep) significantly reduced the extent of heterochromatin formation after confined migration, suggesting an active enzymatic process underlying the increased heterochromatin formation. In particular, enriched phosphorylated (pSer424) HDAC3 nuclear staining suggested activation of HDAC3, potentially due to the mechanical force cells encountered during confined migration. Consistent with this idea, chemical inhibition of HDAC3 significantly reduced heterochromatin formation during confined migration. Interestingly, cells treated with DZnep showed significantly slower migration when compared to control treatment, suggesting the importance of chromatin condensation for efficient migration in 3D environment. Taken together, our research indicates that migration of cancer cells through confined spaces can induce heterochromatin formation, which is expected to both alter the physical properties of the nucleus and to modulate gene expression. These modifications could promote further metastatic cancer progression. Targeting the ability of chromatin condensation upon confined migration may potentially serve as a novel approach in treating metastatic cancers.

P2037/B281

Examining the Role of Chromatin Remodeler-*Skp-1*(snw1) during Cellular Invasion.

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Cellular invasion - the specialized transgression of a basement membrane - is crucial for normal development and a fundamental feature of cancer metastasis. We utilize a unique in *vivo* model, studying the process of anchor cell (AC) invasion into the vulval epithelium during *C. elegans* development as a model for cellular invasion. Previous studies have identified the conserved nuclear hormone receptor transcription factor NHR-67 as crucial to maintaining the AC in G0/G1 cell cycle arrest and have demonstrated the necessity of a histone deacetylase, HDA-1, for the expression of pro-invasive genes. These findings suggest invasion is a differentiated cell behavior requiring genome modification and arrest. To identify additional chromatin modifiers that mediate invasion, we are conducting a tissue-specific RNAi screen using a commercially available ORF library. Thus far, we have screened 70 genes annotated as chromatin regulators and have implicated 6 genes in AC invasion. We have further investigated the gene *skp-1*, of which the mammalian homolog, SNW1, has been studied in the context of oncogenesis. To characterize the function of *skp-1* in AC invasion, we have utilized a cell cycle biosensor providing a visual readout of cell cycle state live based on the ratio of localization of a GFP-tagged fragment of DNA Helicase B in the cytoplasm to nucleus based on CDK2 activity. Our findings demonstrate that RNAi mediated depletion of *skp-1* results in cycling, non-invasive ACs. In addition, we

created an improved RNAi vector, which has increased the penetrance of invasion defects, using available cDNA sequences. We are currently examining the interactions of *skp-1* with the previously identified set of four conserved pro-invasive transcription factors, *fos-1a, egl-43*, *hlh-2*, and *nhr-67* to understand the influence *skp-1* has on the gene regulatory network promoting AC invasion.

P2038/B282

Mechanistic Insights into Cell Motility Under High Hydraulic Resistance.

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Cells in *vivo* encounter a diverse chemo-mechanical micro-environment which influences various cellular processes such as growth, development, disease and motility. This is especially true for a tumor microenvironment where a plethora of cells, extracellular matrix secreted by the cells, macromolecules and interstitial fluid crowd the landscape. These essentially augment the local hydraulic resistance of the microenvironment. It has been previously reported that enhanced extracellular fluidic viscosity (resulting in enhanced hydraulic resistance) leads to augmentation of cell motility. Here we examine the underlying mechanistic insights which play a critical role in augmenting cell motility under high hydraulic resistance. We add a thickening agent to the cell culture medium, resulting in an effective viscosity of 1Pa.s (x 1000 viscosity of water). We found, via pharmacological inhibitions, that the key players of motility under high hydraulic resistance are ion-channels/ pumps, namely, NKCC1 (sodium potassium chloride co-transporter 1), NHE1 (sodium/ hydrogen exchanger 1) and NaK (sodium potassium pump) which influence water permeation. Disruption of cell polarization (via Latrunculin A), which also causes a disruption of polarization of these ion-channels/ pumps, resulted in a significant speed reduction. Interestingly, there is no change in F-Actin expression levels under the influence of high viscous media compared to normal media. We also observed a reduction in actin retrograde flow speed and no change in traction stress in high viscous media compared to the case of normal media. We also investigated calcium dynamics, and found that higher frequencies of calcium oscillations dominate immediately when cells come in contact with high viscous media. These findings have significant implications in shedding light on mechanical aspects of tumor microenvironment, cell motility under significant hydraulic resistance such as in an airway or gastric mucus and cancer cell metastasis.

P2039/B283

Label-free Quantitative Phase Imaging: Applications for Cancer Cell Mechanobiology Studies.

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Mechanical properties of cells are associated with cellular physiological functions by modulating migration, polarization and deformability, and subcellular trafficking. Accordingly, these properties are important for development of cancer. Here we investigated mechanobiological properties on multiple cancer models: head and tumors (primocultures and cell lines) and prostate tumors. Characterization was performed by means of atomic force microscopy, non-coherent quantitative phase imaging, refractive index tomography and confocal microscopy. Cells were further exposed to tubulin-modulating docetaxel. Atomic force microscopy revealed that Young Modulus was increasing with higher invasiveness of cells (determined by engraftment rate in mice, migration speed, scratch and colony-forming assays), more demonstrated on head and neck and prostate cancer cell line model, contrary to a concept that more aggressive cells are of higher compliance. This trend was followed by quantitative

phase imaging - the cell dry mass was increasing with invasiveness pointing out to relation between cell mass density, cytoskeletal composition and cell stiffness. Based on immunofluorescence it was observed that upon docetaxel treatment either actin and tubulin changed from organized to perinuclear shell-like structure in all tested lines followed by dramatic increase of cell stiffness, which again correlated with cell dry mass. Data suggest that cytoskeleton changes affecting mechanobiological properties of cells can be estimated by means of quantitative phase imaging. This work was supported by the Czech Science Foundation GA18-03978S.

P2040/B284

FishATLAS: a Pipeline into Statistical Characterization of Cancer Metastasis in Ewing Sarcoma Zebrafish Xenografts.

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Survival rates of children with various forms of pediatric cancers have seen great improvement in the last 20 years, but unfortunately these gains have not occurred for patient with metastastic cancer. The poor prognosis of metastatic disease urgently calls for research tools that can unravel the complex underpinnings of the metastatic cascade in *vivo,* especially the microenvironmental conditions permissive to induction and growth of metastatic tumors remote from the primary tumor environment. This applies in particular to Ewing sarcoma, a malignant cancer of bone and soft tissue occurring in children, adolescents, and young adults characterized by an oncogenic fusion translocation: *EWS-FLI1*. One-in-three patients with Ewing sarcoma will have metastatic disease at diagnosis, with essentially no avenue to cure. To address this problem we have developed a system in which human Ewing sarcoma cells are xenografted into zebrafish embryos. FishATLAS extracts image data from a large cohort (N>100) of xenografts under different molecular conditions such as basal or knockdown expression levels of the EWS-FLI gene itself or one of its targets. Each fish image is registered by a diffeomorphic transform to a template fish to serve as a coordinate reference frame where FishATLAS determines two crucial pieces of information: 1) Distribution of metastatic spread in conserved, physiologically-relevant microenvironments in *vivo*, and 2) Metastatic microenvironments conserved across different genes and experimental conditions. *EWS-FLI1*'s thousands of genetic targets require a precise map of conserved gene contributions in order to elucidate the complex underpinnings of metastatic disease. Preliminary FishATLAS interrogation of TC32 Ewing sarcoma cell line with or without EWS/FLI1 knockdown shows distinct distributions of cell metastases and primary tumor location. We are expanding this model to patient derived xenografts (PDXs) as a more realistic representation of a human Ewing sarcoma tumor in the FishATLAS pipeline. We are currently in the process of adapting our protocols for this more complex PDX cell injection which includes both cancer and non-cancerous cells.

P2041/B285

Preventive Effect of Proinsulin C-peptide on Pulmonary Vascular Leakage and Metastasis of Melanoma Cells in Diabetic Mice.

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Recently, C-peptide has emerged as a potential candidate for treating various diabetic complications; however, its function against hyperglycemia-induced metastasis is unknown. We hypothesized that hyperglycemia induces metastasis and C-peptide inhibits pulmonary vascular leakage and metastasis in the diabetic mice. VEGF, which is elevated in the lungs of diabetic mice, activated transglutaminase 2 (TGase2) in human pulmonary microvascular endothelial cells (HPMVECs) by sequential elevation of intracellular $Ca2^{+}$ and reactive oxygen species (ROS) levels. VEGF also induced vascular endothelial (VE)-cadherin disruption and increased the permeability of endothelial cells, both of which were prevented by the TGase inhibitors monodansylcadaverine and cystamine or TGM2-specific small interfering RNA. C-peptide prevented VEGF-induced VE-cadherin disruption and endothelial cell permeability through inhibiting ROS-mediated activation of TGase2. C-peptide supplementation inhibited hyperglycemia-induced ROS generation and TGase2 activation and prevented vascular leakage and metastasis in the lungs of diabetic mice. The role of TGase2 in hyperglycemia-induced pulmonary vascular leakage and metastasis was further demonstrated in diabetic $Tgm2^{-/-}$ mice. These findings demonstrate that hyperglycemia induces metastasis, and C-peptide prevents the hyperglycemia-induced metastasis in the lungs of diabetic mice by inhibiting VEGF-induced TGase2 activation and subsequent vascular leakage.

P2042/B286

Invadopodia Facilitate the Cooperative Invasion of Breast Cancer Cells.

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Invasive breast cancer is, in most cases, an uncurable disease while localized tumors are clinically manageable. We and others have shown that to negotiate the structural barrier imposed by the surrounding extracellular matrix (ECM) in the healthy tissue, invasive cancer cells utilize dynamic protrusions enriched in matrix metalloproteinases to remodel collagen fibers. While it is accepted that these protrusions, called invadopodia, contribute to the invasion of E-cadherin (-) cells, invading individually, whether and which cell assemble invadopodia during cohesive multicellular invasion of E-cadherin (+) remains unknown. So far, in an effort to limit cancer cell invasion and metastasis, most of the attention has been drawn to inherently invasive cells, frequently disregarding non-invasive cancer cells. However, tumors are highly heterogeneous, and contain cancer cell subpopulations with varying degrees of invasion potentials. Recently, it was observed that in 3D spheroid invasion assay, invasive cells create paths inside the ECM, into which non-invasive cells may subsequently migrate, a phenomenon termed “cooperative invasion”. Moreover, the possibility that cooperation among heterogeneous cancer cells might contribute to the dissemination of non-invasive cells in tumors is yet to be tested. Using the 3D spheroid invasion assay, our preliminary results in both human and mouse cell lines demonstrate that invadopodia are primarily assembled by cells located at the tip of invasive strands. This was observed in all types of strands, including both E-cadherin (-) strands and E-cadherin (+), cohesive strands. Furthermore, we found that coculture of non-invasive cells, together with invasive

cells that assemble invadopodia, enables the cooperative invasion of these two cell types. Hence, invadopodia are necessary only for path creation, but not for path following. To test if migration and lung colonization of non-invasive cells can be mediated by invadopodia during cooperative invasion, we are using preclinical mouse models of human and mouse mammary carcinoma. We propose that cooperative invasion is a new mechanism by which heterogeneous breast cancer cell populations can disseminate. In conclusion, targeting invadopodia may be a potent strategy to inhibit dissemination of both individually, as well as collectively migrating cells.

P2043/B287

Understanding the Coordination of Invadopodia with Cell Cycle Progression.

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Tumor cell invasion comprises cell migration and assembly of invadopodia, which are protrusions capable of degrading the extracellular matrix (ECM). The effect of cell cycle progression on invadopodia has not been elucidated. In this study, using invadopodia- and cell cycle- fluorescent markers, we show in 2D and 3D cultures, as well as in *vivo*, that breast carcinoma cells assemble invadopodia and invade into the surrounding ECM preferentially during the G1 phase. Interestingly, during strand invasion in 3D spheroids, the leader cells are enriched in G1 and upon transitioning to the S and G2 phases, they are replaced by another cell in G1 phase. Furthermore, cells synchronized in G1 phase exhibit significantly higher ECM degradation compared to the cells synchronized in S phase. Consistent with this, the expression (MT1-MMP, cortactin) and localization (Tks5) of invadopodia components are elevated in G1. The cyclin-dependent kinase inhibitor (CKI) $p27^{kip1}$ localizes to the sites of invadopodia assembly. Over-expression and stable knockdown of $p27^{kip1}$ affect invadopodia turnover and ECM degradation. Taken together, these findings suggest that expression of invadopodia components, as well as invadopodia function, are linked to cell cycle progression and that invadopodia is controlled by cell cycle regulators. Our results caution that this coordination between invasion and cell cycle must be considered when designing effective chemotherapies.

P2044/B288

Cells in Intermediate States during EMT Are Characterized by Specific Geometrical and Mechanical Intra-cellular Architectures.

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The epithelial to mesenchymal transition (EMT) allows mammary breast cancer cells to dissociate from the primary tumour and form metastases. Recent works have revealed that the *dangerous* cells that have acquired augmented capacity to migrate and transdifferentiate into several cell types are in an intermediate stage between the epithelial and the mesenchymal state. It is now required to unravel the molecular and cellular mechanisms responsible for the acquisition of these properties and to identify early and specific structural markers characterizing these stages. Here we induced the expression of ZEB1, a transcription factor responsible for EMT initiation, to generate intermediate stages of EMT in

human mamary epithelial cells and stimulation with TGFβ to push further the transition to the mesenchymal state. We measured and compared the architecture, internal organisation and mechanical properties of each state. We found that the lack of inter-cellular cohesiveness in intermediate and later stages of EMT can be detected early by microtubule destabilisation and the repositioning of the centrosome from the cell junction to the cell center. Consistent with their high migration velocities, we found that cells in intermediate state of EMT were in a low tensional state compared to epithelial and mesenchymal cells. The high contractility of mesenchymal cells powered a retrograde flow pushing the nucleus away from cell adhesion to the extra-cellular matrix. These measures revealed how defined structural and mechanical rearrangement in intermediate stages of EMT confered them specific dissociation and migration properties that distinguish them from epithelial and mesenchymal states.

P2045/B289

E-cadherin Is an Invasion Suppressor, Survival Factor, and Metastasis Promoter in Multiple Models of Breast Cancer.

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The loss of E-cadherin (E-cad) has been linked to increased invasion of cancer cell lines and has been inferred to promote metastasis, leading to its classification as a tumor, invasion, and metastasis suppressor. However, about 90% of breast cancers retain E-cad expression in both the primary tumor and distant metastases. In this study, we test the requirement of E-cad for metastasis using mouse models of luminal and basal breast cancers that allow for an inducible deletion of E-cad. Loss of E-cad increases tumor invasion and dissemination in both 3D organotypic *ex vivo* assays and in *vivo* assays. In contrast, E-cad loss strongly inhibits metastasis. Careful analysis of intermediate stages of metastasis revealed that despite an increase in local invasion, E-cad loss is associated with a decrease in migratory persistence, decrease in circulating tumor cell counts, decrease in tumor seeding potential, and decrease in metastatic outgrowth, collectively causing a strong decrease in metastasis. Mechanistically, we found a TGFβ-dependent enrichment of reactive oxygen species specifically within the disseminated E-cad- cancer cells, frequently leading to their apoptosis. Significantly, colony formation of E-cad-negative cells was rescued by treatment with a TGFβ-receptor inhibitor, an anti-oxidant, or an apoptosis inhibitor. These results suggest that E-cad acts as a survival factor during the detachment, systemic dissemination, and seeding phases of metastasis in these models of breast cancer.

P2046/B290

E-cadherin Promotes Cell Proliferation in Breast Cancer.

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E-cadherin promotes cell proliferation in breast cancer Gabriella Russo, Michelle Karl, Meng-Horng Lee, Denis Wirtz Currently classified as a tumor suppressor gene, E-cadherin (Ecad) is a widely studied protein for its association with the epithelial to mesenchymal transition (EMT). Functioning as a cell-cell adhesion molecule, the loss of Ecad is a marker of cells undergoing a transition from a more epithelial phenotype to a more migratory and invasive one, resulting in the early dissemination of cancer cells from the primary tumor. However, recent clinical data for breast cancer suggest that Ecad may function as an oncogene in certain subtypes. This apparent contradiction may be due in part to the 2D *in vitro* systems widely used to study EMT processes. As the effect of Ecad loss on cell behavior can vary greatly

depending on the model used, we studied the role of Ecad in cancer cell proliferation and invasion/migration using a more physiologically relevant in *vitro* system developed in our lab. This novel two-compartment 3D organoid model contains both a basement membrane and stromal collagen I matrix allowing us to study the first step in the metastatic cascade. This 3D in *vitro* model suggests that Ecad induces hyper-proliferation in breast cancer cells through activation of the ERK signaling pathway. This result was validated in multiple in *vivo* mouse models. Depletion of Ecad and pharmacological inhibition of ERK in Ecad-expressing cells both depress tumor growth in preclinical models of breast cancer.

P2047/B291

Ovarian Carcinosarcoma with Epithelial-mesenchymal Transition.

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Ovarian cancer is one of the most common cancers in females. In 2018, nearly 300,000 new cases were reported [(1)]. Specifically, a portion of these incidences are attributed to malignant mixed mesodermal tumors such as ovarian carcinosarcoma, that is a rare and aggressive cancer of the ovary, characterized by biphasic histology, with both carcinomatous and sarcomatous elements. Early detection and therapeutic approaches have been unsuccessful as the origin and pathological process of this specific cancer is poorly understood[(3)]. Epithelial-mesenchymal transition (EMT) is a physiological process observed during embryogenesis also involved in cancer cell invasion and metastasis[(2)]. Five patients were identified with advanced-stage ovarian carcinosarcoma in our hospital from 2000 to 2018. Age ranged from the 50s to 70s with common clinical symptom was abdominal pain. The size of the tumor was 10 to 20 cm. The cancer component was mostly endometrioid carcinoma. The sarcoma component was osteosarcoma in 3 cases and homologous sarcomatous in 2 cases. Regarding staining, E-cadherin was partially negative, and N-cadherin was positive. Ideally, snail should be positive, but the result showed negative. Our result did not support the EMT transition hypothesis in carcinosarcomas genesis.

P2048/B292

Evaluation of EMT Markers in Cadmium-treated Mammary Epithelial Cells.

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The epithelial-mesenchymal transition (EMT) is a transdifferentiation process from an epithelial to a mesenchymal phenotype. During EMT occurs changes in expression and/or activation of adhesion proteins, kinases, transcription factors and hydrolases. Breast cancer is the most frequent neoplasia and the leading cause of death in women worldwide. In addition to the well-known classical and genetic risk factors for development and poor prognosis of breast cancer, exposure to environmental pollutants such as cadmium (Cd) are currently been investigated. In this context, we aimed to evaluate the effects

of chronic cadmium exposure in mammary epithelial cells, the expression of the canonical EMT markers such as morphological changes, E-cadherin and vimentin levels, kinases activity and cell migration. MCF10A cell line cultures were maintained through 28 days with different Cd concentrations (0.5, 1.5, 2.5, 5 and 10 µM). We visualized cell morphology using rhodamine-phalloidin in an epifluorescence microscope. Expression levels of E-cadherin and vimentin were evaluated by Western blot. Finally, wound-healing assays were performed to assess cell migration. Our data showed that after prolonged cadmium treatment, morphological changes and stress fibers formation depends on the Cd doses. We also observed a decrease in E-cadherin levels, increased vimentin expression, and gain of cell migration in MCF10A cell line. Together, our data suggest that chronic exposure to different cadmium concentrations promotes EMT features and enhanced migratory properties related to breast cancer progression in MCF10A epithelial breast cells.

P2049/B293

G1p3-induced Mtros Elicits Retrograde Signaling Alters Nuclear Gene Expression of Cav1 and Scn-1 to Promote Breast Cancer Cell Migration.

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Metastasis is the leading cause of deaths in > 90% of breast cancer patients. Recently, we reported that G1P3 (IFI6/ISG6-16), an interferon-stimulated gene, promotes breast cancer metastasis. Although G1P3 was suggested as a mitochondrial protein with a role in elevating mitochondrial reactive oxygen species (mtROS), its submitochondrial localization was inconclusive. Therefore, pure mitochondria were isolated using anti-TOM22 microbeads (Miltenyi Biotec) and sub-fractionated by differential centrifugation. Western blot analysis identified localization of G1P3 in the inner mitochondrial membrane (IMM) fractions. Additionally, sodium carbonate flotation assay that releases peripheral membrane proteins failed to extract G1P3 and intact mitochondria protected G1P3 from trypsin digestion, confirming its IMM localization. Based upon these results and G1P3's ability to promote cancer cell migration by mtROS levels, we hypothesized that retrograde signaling elicited by G1P3-induced mtROS alter nuclear gene expression to confer migratory potential in breast cancer cells. This hypothesis was tested by comparative gene expression and pathway analysis of G1P3-expressing (MCF-7^{G1P3}) and nonexpressing cells ($MCF7^{Vector}$). In agreement with its effects on actin remodeling to promote migratory structures, genes involved in regulation of wound healing, epithelial to mesenchymal transition (EMT), cytoskeleton organization, and oxidative response pathways were enriched in $MCF7^{G1P3}$ cells. In quantitative RT-PCR analysis, in $MCF7^{G1P3}$ cells, Scinderin1 that sever F-actin was downregulated by 5-fold and Caveolin 1, a regulator of endocytosis was upregulated in by 4-fold ($p \leq 0.05$). Moreover, MitoTEMPO, a mitochondrial specific ROS scavenger, reversed the expression of Caveolin 1 and Scinderin in MCF-7^{G1P3} cells. The direct role of G1P3 in regulating Cav1 expression was assessed using a specific siRNA. In immunoblot analyses, Cav1 protein was 3-fold higher in MCF-7^{G1P3} cells that was abrogated by siG1P3 or by mitoTEMPO with a concurrent abrogation of mtROS levels. Taken together, our results demonstrate a role for G1P3-induced mtROS in eliciting retrograde signaling to alter nuclear gene expression of Cav1 and Scn 1 to promote breast cancer cell migration.

P2050/B294

FAK and Src: Regulators of EMT-related Transcription Factors in Mammary Epithelial Cells Stimulated with Leptin.

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The Epithelial-Mesenchymal Transition (EMT) is a reversible cellular trans-differentiation program from epithelial to a mesenchymal phenotype, where specific transcription factors such as Snail, Slug, Zeb and Twist are activated. Leptin is a hormone secreted mainly by adipose tissue, and in a lesser extent by normal and tumorigenic mammary epithelial tissue, its main function is regulating the appetite, however, the overexpression of leptin and its receptor, ObR, has been associated with tumor progression in breast cancer. However, the molecular mechanism that underlies these events is not fully understood; nevertheless, the activation of different signaling pathways appears to be essential. The aim was to determine the role of Src and FAK in the expression of EMT-related transcription factors in MC10A and MCF7 mammary epithelial cell lines stimulated with leptin. Specific events occurring during EMT were also evaluated in the presence or absence of the kinases's chemical inhibitors PP2 and PF-573228. For instance, we tested the expression and subcellular localization of the EMT-related transcription factors Twist and β-catenin, by western blot and immunofluorescence. We also evaluated the secretion and activation of matrix metalloproteases (MMP-2 and MMP-9) by gelatin zymography. Invasiveness properties of leptin-stimulated cells were determined by invadopodia formation assays, and by the transwell chamber method. Our results showed that leptin promotes EMT through Src and FAK activation, which leads to the secretion and activation of MMP-2 and MMP-9, invadopodia formation and cell invasion in MCF10A cells. In conclusion, our data suggest that leptin promotes an increase in the expression levels of Twist and β-catenin, the secretion of MMP-2, MMP-9, the invadopodia formation and invasion in MCF10A cells in a Src and FAK-dependent manner.

P2051/B295

Inhibition of the Fractionated Ionizing Radiation-induced Epithelial to Mesenchymal Transition Signal by Socs1 through Ros Regulation.

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Fractionated ionizing irradiation (FIR) is widely employed for the treatment of diverse types of cancers. However, cancer cells often develop resistance to the FIR therapy given at multiple low doses. Epithelial to mesenchymal transition (EMT) is recognized as a process associated with the therapy-induced resistance of cancers. The present study was conducted to investigate the mechanism of FIR-induced EMT response and the associated signaling pathways to understand molecular basis of the FIR-mediated radioresistance. In HCT116 colorectal cancer cell lines, FIR induced intracellular reactive oxygen species (ROS) and changes indicative of EMT features such as down-regulation of E-cadherin along with up-regulation of Twist and Snail. Anti-oxidant pretreatment abrogated the FIR-induced ROS generation and significantly attenuated EMT response, indicating that FIR-induced EMT is mediated by ROS signal. Mechanistically, the FIR-induced ROS-mediated EMT process appeared to proceed through Akt --> Src -->Erk signaling pathways. As suppressor of cytokine signaling 1 (SOCS1) has been reported for the anti-ROS and anti-EMT action, the transduction of SOCS1 into HCT116 cells inhibited the FIR-induced changes for EMT with thioredoxin (Trx)1 up-regulation. Notably the shRNA-mediated Trx1 ablation in SOCS1-

transduced cells restored the FIR-induced ROS and EMT features. Together, the results of the present study indicate that ROS signal acts as a mediator of the FIR-induced EMT response, which suggest a potential anti-tumor function of SOCS1 in colon cancer cells through the regulation of ROS/Trx1 axis.
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Tumor Microenvironment: Immune Microenvironment

P2052/B296

Contact-mediated Macrophage Mitochondrial Transfer in Breast Cancer Cell Metastasis.

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The presence of macrophages in the tumor microenvironment has been correlated with poor prognosis and increased tumorigenicity in many cancer types. However, the macrophage-tumor cell interactions that contribute to this increased tumorigenicity remain unclear. Recently, we discovered that macrophages transfer cytoplasm to melanoma cells in *vivo and* the majority of the recipient melanoma cells disseminate from the primary transplantation site. These data suggest macrophages transfer a "package" of cytoplasm containing molecules that may influence the behavior of recipient cells. However, central questions that remain are: 1) what is identity of the transferred molecules? and 2) what is the mechanism underlying this transfer? Here, we report that mitochondria are transferred from macrophages to cancer cells and this occurs through a cell contact-dependent mechanism. Most examples of mitochondrial transfer in the literature show population-level phenotypes, thus it is still unclear what is happening in recipient cells on a cell-biological level and whether the observed phenotypes can be attributed to the receipt of functional mitochondria. By genetically labeling mitochondria in primary human macrophages and human breast cancer cells, we found that donated mitochondria do not incorporate into the host cancer cell mitochondrial network, but instead persist as a separate, intact population. Furthermore, we discovered that recipient cancer cells exhibit an increased proliferative index. Interestingly, our data suggests this phenotype may not be due to the receipt of functional mitochondria as these donated mitochondria have a reduced membrane potential yet they can be visualized in recipient cells several hours post-transfer suggesting they are not being degraded. Taken together, our results indicate the transfer of macrophage mitochondria elicits an increase in proliferation in recipient cancer cells. We aim to explore this further by investigating the following possibilities: 1) donated mitochondria are sufficient to trigger a biological response in the recipient cell or 2) additional cellular contents are co-transferred and have instructive capabilities. Towards these ends, we are directly testing the function of transferred mitochondria by exogenously injecting purified mitochondria into cancer cells. In addition, we are characterizing the function of transferred mitochondria using biosensors and in *vivo* models. Future work will address whether mitochondria or other transferred molecules provide essential cues that drive increased tumorigenesis. These studies will more clearly define how donated molecules can lead to functional changes in recipient cells and determine how macrophage and tumor cell interactions contribute to metastasis.

P2053/B297

Macrophage-dependent Mitochondrial Dynamics in Cancer.

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Metastasis is not a one-time event, but a progressive process in breast cancer, generally occurring multiple times over the course of a patient's life. Since metastasis is the main cause of cancer-related deaths, it is imperative to develop treatment strategies to prevent progression of the disease after initial diagnosis. In addition to genetic changes in tumor cells, a major determinant of tumor cell behavior stems from local interactions within the tumor microenvironment. With the initial successes of immunotherapies, a wealth of studies have defined roles for many of the tumor microenvironmental immune components in controlling tumor cell behavior. It has become clear that intercellular communication strongly underlies therapeutic resistance and tumor relapse. One immune cell type known to frequently communicate with tumor cells is tumor-associated macrophages, which can both promote and block tumorigenesis. This plasticity is due to the ability of macrophages to differentiate into distinct states in response to environmental cues. Recently, we discovered an unconventional form of cell-cell communication in which human macrophages transfer mitochondria to breast cancer cells. Cancer cells that receive macrophage mitochondria exhibit increased proliferation, a critical step in metastasis. To understand how mitochondrial transfer is regulated, we altered macrophage differentiation by inhibiting the Notch pathway. Inhibiting the Notch pathway resulted in macrophage differentiation to a pro-tumorigenic state and increased macrophage mitochondrial transfer to cancer cells. We are currently testing how the Notch pathway regulates mitochondrial intercellular dynamics in macrophages, and how altering intercellular dynamics affects mitochondrial transfer to cancer cells. These studies will reveal valuable insight into how cellular differentiation influences intracellular organelle dynamics. Furthermore, given that Notch inhibitors are currently under clinical pursuit, these studies will provide critical information on the efficient use of these inhibitors to treat cancer patients.

P2055/B299

Progesterone Receptor Promotes Degradation of Stat2 to Inhibit the Interferon Response in Breast Cancer.

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The progesterone receptor (PR) is a nuclear receptor that is activated in response to its ligand, progesterone. Traditionally, PR exerts its effects following ligand activation by translocating to the nucleus and binding to DNA where it affects the transcription of a variety of genes involved in growth, survival and proliferation. Our lab, using microarray data combined with Gene Set Enrichment an alysis, has identified a novel subset of genes that have altered expression following PR activation. These genes are primarily involved in interferon signaling—a pathway normally utilized in response to viral infection. Our data show that many genes that are normally activated in response to interferon signaling (interferon stimulated genes, ISGs) are repressed when PR is activated by its ligand. As much of the past research focus has been on PR's ability to promote transcription of its target genes during pregnancy/mammary gland development, little is known of PR's role as a transcriptional repressor. In attempting to elucidate a mechanism by which PR exerts this effect, we have investigated crosstalk of PR with canonical interferon signaling proteins and have found that PR interacts with these proteins and significantly reduces their ability to become activated. In particular, a new finding from our lab has found that PR promotes the ubiquitination and degradation of STAT2-an indispensable protein involved

in the interferon signaling cascade. Without STAT2, interferon signaling is effectively abrogated. Experiments are currently underway to identify precisely how PR activation results in turnover of the STAT2 protein, and how this translates to inhibition of the interferon response. Our lab is interested in identifying the consequences of this PR-dependent inhibition of efficient type I interferon signaling in the context of breast cancer. Evasion of the immune system is considered a Hallmark of Cancer and our preliminary data suggest a potential mechanism by which breast cancer is able to accomplish this. Activation of type I interferon signaling is an early step in marking tumors for immune clearance. By repressing ISG protein expression, it is possible that these tumors are able to avoid detection by host immune cells leading to tumor establishment and subsequent progression. This novel role of PR offers insight that could aid in improving upon established estrogen only-based therapies for prevention and treatment of hormone dependent breast cancers.

P2054/B298

Upregulation of the Nlrc4 Inflammasome Contributes to Poor Prognosis in Glioma Patients.

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Inflammasomes which are majority complex of NLRs family with caspase-1 is strongly associated with various tumor development. However, among the NLRs family, role of NLRC4-mediated inflammasome to glioma is unclear. in this study, we identified that the NLRC4-mediated inflammasome is associated with poor prognosis of glioma patients from TCGA data. Immunohistochemistry was shown that NLRC4 and caspase-1 were co-expressed in astrocytoma cells in 11 tissue of glioma patients. in addition, several key proteins which are related with NLRC4-mediated inflammasome pathway were also associated with poor prognosis of glioma patients in TCGA dataset. Our results suggest that the upregulation of the NLRC4-mediated inflammasome contributes to a poor prognosis for gliomas and presents a potential therapeutic target and diagnostic marker.

P2056/B300

The CXCL5/CXCR2 Axis Is Sufficient to Promote Breast Cancer Colonization during Bone Metastasis.

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Bone is one of the most common sites for metastatic tumor growth across cancers, including breast cancer. Cancer cells that travel through the vasculature and invade a new tissue can remain in a quiescent, non-proliferative dormant state for years until the cancer cells are induced to colonize the metastatic site. This switch from dormancy to colonization is the rate-limiting step for bone metastasis. To model the cancer cells as they colonize, we developed an *ex vivo* co-culture system of mouse bones and cancer cells and identified culture conditions with high or low cancer cell proliferation and invasion using healthy bones or cancer primed bones. To identify the factors that promote cancer cell colonization, we profiled cytokines, chemokines, and growth factors from conditioned media and identified the chemokine CXCL5 as a candidate to induce metastatic colonization. Additional studies using CXCL5 recombinant protein further suggest that CXCL5 is sufficient to promote breast cancer cell

proliferation and colonization in bone, while inhibition of its receptor CXCR2 with an antagonist blocks proliferation of metastatic cancer cells. This study suggests that CXCL5 and CXCR2 inhibitors may have efficacy in treating metastatic bone tumors that are dependent on the CXCL5/CXCR2 axis.

P2057/B301

Mesenchymal Stromal Cell-derived CXCL16 Promotes Proliferation and Migration of Gastric Cancer Cells by Inducing STAT3-mediated Expression of Ror1.

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Bone marrow-derived mesenchymal stem or stromal cells (MSCs) have been shown to be recruited to various types of tumor tissues, where they interact with tumor cells to promote their proliferation, invasion, and metastasis, depending on the types of the tumor cells. We have previously shown that Ror2 receptor tyrosine kinase and its ligand, Wnt5a, are expressed in MSCs, and activated Wnt5a-Ror2 signaling in MSCs induces expression of CXCL16, which in turn promotes proliferation of co-cultured MKN45 gastric cancer cells via CXCL16-CXCR6 axis. However, it remains unknown how CXCL16 regulates proliferation of MKN45 cells. Here, we show that siRNA -mediated knockdown of *CXCL16* in MSCs suppresses not only proliferation, but also migration of co-cultured MKN45 cells. We also show that MSC-derived CXCL16 or recombinant CXCL16 induces expression of Ror1 through activation of STAT3 in MKN45 cells and that up-regulated expression of Ror1 plays an important role in mediating proliferation and migration of MKN45 cells in *vitro*. Moreover, it was found that transplantation of MKN45 cells along with MSCs in nude mice promoted tumor formation in a manner dependent on expression of Ror1 in MKN45 cells, and that anti-CXCL16 neutralizing antibody suppressed tumor formation of MKN45 cells co-transplanted with MSCs. Collectively, these results indicate that CXCL16 derived from MSCs induces expression of Ror1 through activation of STAT3 pathway in MKN45 cells, eventually leading to promotion of tumor formation.

P2058/B302

Molecular Delineation of Ccl18-elicited Membrane Organelle Interactions during Breast Cancer Migration and Invasion.

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Mitochondria are dynamic organelles with context-dependent subcellular redistribution in response to extracellular cue changes, although the underlying mechanisms remain largely elusive. The ARF6 GTPase and its signaling pathway involving ACAP4 promote cell invasion via Grb2-adaptored integrin recycling (Yu et al., 2011. JBC). Our recent work demonstrated that ACAP4 acetylation elicited by CCL18 regulates dynamic membrane-membrane remodeling associated with breast cancer cell migration (Yuan et al., 2018. JMCB). Using functional proteomics screen, we identified a novel regulator of ACAP4 named Acapin. Our biochemical characterization showed that Acapin binds and inhibits the GAP activity of ACAP4. Interestingly, CCL18 stimulation elicited the recruitment of Acapin to thelamellipodium membrane in addition to mitochondria polarization toward the lamellipodium. We are currently carrying out spectral imaging analyses to visualize how ACAP4, ARF6 and Acapin interact and orchestrate dynamic organelle contacts during CCL18-elicited breast cancer cell migration and invasion.

P2059/B303

CCL18-elicited Segregation of Mitochondria Drives Breast Cancer Cell Migration and Invasion.

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Tumor metastasis represents the main cause of cancer-related death. Our early study showed that chemokine CCL18, secreted from tumor-associated macrophages, regulates breast tumor metastasis. Our recent work demonstrated that ARF6 GTPase-activating protein ACAP4 regulates CCL18-elicited breast cancer cell migration via the acetyltransferase PCAF-mediated acetylation (Yuan et al., 2018, JMCB). Using triple negative breast cancer cells as a model, we show that mitochondria actively segregate toward leading edge lamellipodia in response to CCL18 stimulation, thereby increasing local mitochondrial mass and relative ATP concentration. Since the organization of membrane-bound organelles allow for the separation of incompatible biochemical processes, we are currently carrying out spectral imaging analyses to understand how CCL18-elicited organelle communication orchestrates directional cell migration. In addition, we combine live cell imaging with chemical probes to delineate the molecular mechanisms underlying CCL18-elicited organelle interactome during directional cell migration.

P2060/B304

The Role of PTPRJ in Neutrophil Polarization in the Tumor Microenvironment.

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Neutrophils are highly infiltrative into many solid tumor types and a high neutrophil-to-lymphocyte ratio is an indicator of poor patient prognosis. However, increasing evidence indicates that neutrophils are highly plastic cells that can have either tumor promoting or inhibiting capabilities. This polarization can be influenced by the tumor microenvironment, but the intracellular signaling changes responsible for neutrophil pro- or anti-tumor polarization are not well understood. Therefore, we sought to identify a novel pathway that modifies neutrophil polarization in the tumor microenvironment. To identify putative transcriptional targets involved in neutrophil polarization in cancer, we have conducted Translating Ribosomal Affinity Purification Sequencing (TRAPseq) using the zebrafish model organism. Zebrafish are an ideal model for studying the role of neutrophils in early cancer initiation and progression due to their optical transparency and high fecundity. In accordance with human clinical data, our lab has previously shown that neutrophils are tumor promoting in these zebrafish models. Using TRAPseq in our established zebrafish HRas-transformed keratinocyte cancer model, we identified upregulation of targets specifically in the neutrophil population. PTPRJ, a transmembrane phosphatase, was found to have a 5-fold increase in expression in the cancer model over wild-type zebrafish. In mice, PTPRJ has previously been shown to modify neutrophil superoxide production and chemotaxis, processes that are both important to neutrophil function in the tumor microenvironment. Therefore, we have chosen to evaluate the role of human neutrophil PTPRJ in the context of cancer. As genetic manipulation of primary human neutrophils is not possible, we have generated PTPRJ knockout lines in the HL-60 neutrophil-like cell line using CRISPR/Cas9. Using these lines, we have shown that PTPRJ has an effect on neutrophil chemotaxis in an in *vitro* microfluidic device. Furthermore, we have identified a published PTPRJ agonist to evaluate phosphatase activity in primary human neutrophils, which has previously not been possible due to their short lifespan *ex vivo*. Finally, we have generated a PTPRJ-

knockout zebrafish line using CRISPR/Cas9 to evaluate neutrophil function in an in *vivo* tumor xenograft model. This study demonstrates the power of combining TRAPseq technology with the zebrafish model system to identify transcriptional targets that are altered in disease backgrounds.

P2061/B305
Characterizing the Inflammatory Consequences of Failed Mitoses.
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Cytoplasmic self-DNA (cyDNA) accumulation has been proposed to play a causative role in multiple pathologies including autoimmune disorders, cancer and possibly aging by activating innate immune pathways. Specifically, the cGAS-STING-Interferon (IFN) pathway has been identified as playing a critical role in mediating cellular and tissue response to cyDNA. cyDNA is thought to accumulate through multiple routes, including mechanical rupture of micronuclei, nuclear budding and export of DNA fragments via nuclear pores. Anti-microtubule drugs, such as Vinca alkaloids and Taxanes, represent a cornerstone of cancer chemotherapy. They kill cancer cells in culture via perturbation of mitosis, and are often termed “anti-mitotics”. However, other drugs that target mitosis-specific proteins such as inhibitors of Eg5/Kif11, Aurora kinases a and B, and Polo-like kinase 1 have proven to be ineffective for cancer treatment. I hypothesized that anti-mitotic drugs might differ in their ability to trigger cGAS-STING signaling after a failed mitosis, and this might help to explain their observed differences in clinical efficacy. To test this I performed a pharmacological screen of a diverse set of anti-mitotics and systematically measured their ability to induce micronuclei, cyDNA and IFN. To measure IFN production in a physiologically relevant manner, I developed a high-throughput co-culture assay in which engineered immune cells report on the concentration of extracellular IFN secreted by drug treated cancer or stromal cells. I found that some anti-mitotic drugs generate more IFN than Taxanes, and others much less. My data point to a role for mitotic kinases in regulating cGAS activation following a failed mitosis. Manipulation of mitotic kinases also allowed me to distinguish alternative pathways by which DNA enters the cytoplasm in response to anti-mitotic vs. DNA-damaging drugs.

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P2062/B306
Identification of Genome-wide Molecular Characteristics in Ameloblastoma: Role of *Tlr2* in Ameloblastoma Cells.
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Ameloblastoma is a rare odontogenic benign tumor accounting for less than 1% of head and neck tumors. Despite the reports regarding whole genomic sequence information from patients with ameloblastoma, gene expression profiles in ameloblastoma are still obscure. The present study is the first to demonstrate novel genome-wide molecular characteristics in ameloblastoma, which is a rare neoplasm, yet also the most frequent in odontogenic tumors. Our high-resolution comparative genomic hybridization <aCGH> showed wide-range of minimal somatic copy number aberrations <CNAs> as well as distinct gene expression pattern in ameloblastoma, in which oncogenic gene signatures including Kras-responsive genes and EGFR-related genes are significantly activated. Of note, the altered gene

expression partly paralleled the CNAs detected. Interestingly, expression of toll-like receptor 2 <TLR2> is highly upregulated in ameloblastoma tumors and in the ameloblastoma cell line AMU-AM1. Importantly, TLR2 knockdown suppressed the activation of *KRAS*-responsive genes as well as inflammatory response genes. Furthermore, TLR2 knockdown significantly increased caspase-3 activity. In summary, our findings uncovered possible genome-wide molecular characteristics and showed the significant involvement of TLR2 in the cellular biological properties of ameloblastoma. Our data might be a help to further understand the molecular pathogenesis of ameloblastoma in future clinical biological studies.

P2063/B307

Understanding the Human Equilibrative Nucleoside Transporter (slc29a1): New Modalities of Regulation and Therapeutics.

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The nucleoside transporter, hENT1 (SLC29A1), is responsible for the flux of endogenous nucleosides such as adenosine, across the plasma membrane and is therefore an important component of the cellular purinome (Dos Santos Rodrigues et al 2014). ENT1 is also a major route of entry for nucleoside analog drugs, such as gemcitabine, which are used widely in chemotherapy and anti-viral treatments. While we have made significant advances in understanding the complex regulation of ENT1 (Grane-Boladeras et al 2019) including identification of novel interactors (Guo et al. 2019), a deeper understanding of the cell biology of hENT1 is needed in order to develop more specific and effective nucleoside analog based chemotherapeutics. Moreover, drug-resistance is a significant clinical challenge and one way to address this challenge is by developing novel therapies that combine well-established pharmacology (nucleoside analog drugs) and innovative biophysical interventions, such as ultrasound microbubble (USMB) treatments. To this end, we are using the pancreatic tumour cell lines PANC1 & BXPC3 which express high levels of hENT1 and which are differentially sensitive to gemcitabine, a front-line drug in the treatment of pancreatic cancer. Drug uptake requires the presence of functional ENT1 in the plasma membrane. Therefore, understanding the nature of the cellular context of ENT1 is essential in defining factors that influence drug uptake (and therefore efficacy). We have determined that ENT1 is localized to caveolin-1 rich microdomains which are implicated in regulated internalization. Furthermore, we have discovered that gemcitabine treatment selects for a sub-population of drug-resistant cells which exhibit behaviours and cell biology suggestive of transformed and potentially metastatic phenotypes. We have confirmed that drug plus USMB is cytotoxically additive at a single time point (Mariglia et al. 2018) and now show that the most effective combination modality for cytotoxicity is drug treatment followed by USMB. These findings provide the basis for the further studies aimed at refining novel combination therapeutics based on well established nucleoside analog drugs plus innovative biophysical interventions, while also defining the underlying mechanistic aspects of the cellular responses.

P2064/B308

Lipocalin-2 Regulates Dna Damage Response Pathway Upon Genotoxic Stress.

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Genotoxic are known to cause DNA damage and subsequently inducing cell death. In response to DNA damage, cells activates the DNA damage response (DDR) which coordinate a complex interaction of

downstream pathways to determine cell fate, including coordination of DNA repair, cell cycle arrest and apoptosis. In cancer, treatments commonly use DNA-damaging drugs; however, some cancer cells are able to survive this process. This resistance has been correlated with the expression of Lipocalin-2 (Lcn2). Lcn2 is a 25kDa secreted glycoprotein known for its role in the cellular transport of lipophilic molecules such as fatty acids, iron, and steroids. Lcn2 play critical roles in several pathological organ conditions and particularly in cancer condition, it has been demonstrate that it facilitates tumorigenesis by promoting survival, growth, and metastasis. However, the mechanisms controlled by lcn2 to ensure cell survival are not known. We used kidney cells expressing Lcn2, invalidated for its expression or overexpressing Lcn2. We observed that cells expressing Lcn2 increased cell survival to genotoxic drugs. Also, we observed that kidney cancer cells expressing Lcn2 are more prone to developed resistance to genotoxic drugs as opposed to kidney cancer cells lacking Lcn2 expression. Hence, Lcn2 appears to participate to cellular mechanisms responsible for genotoxic stress survival. To evaluate whether Lcn2 may regulate the DDR, we have monitored the effect of Lcn2 knockdown (KD) on the activation status of DDR proteins. We observe that Lcn2 KD decreases genotoxic induced ATM phosphorylation, and P53 subsequent activation. This resulted in an increase of the blockage of the cell cycle in S-phase normally observed upon genotoxic stress. Interestingly, using comet assay experiment, we showed that Lcn2 does not seem to impact the amount of DNA breaks induced by genotoxic stress neither on the expression of γH2AX, a marker of DNA repair. Together, these results suggest that Lcn2 expression allows cell survival when exposed to genotoxic stress probably through an increase of the DDR pathway activity.

P2065/B309

Combinatorial Application of Doxorubicin and Delta-tocotrienol Induces Apoptotic and Autophagic Death on Colorectal Cancer Cells.

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Doxorubicin (Dox) has been widely used in chemotherapy for treating various cancers, including the colorectal cancer. However, the effectiveness of Dox is often limited by high-dose toxicity and tendency of resistance development. As an approach to augment the effectiveness of Dox, delta-tocotrienol (δT3) was applied in a combination aiming to study the anticancer effects on colorectal cells. The scope of this study included the in *vitro* evaluations of antiproliferative effects, morphological changes, DNA damages, cell cycles and protein expression profiles in relation to apoptosis and autophagy. The combined treatment of delta-tocotrienol and doxorubicin (δT3 + Dox) resulted pharmacological synergisms on a couple of colorectal cancer cell lines and particularly exerted significant anti-clonogenic survivals on Caco-2 and SW48 cells even at low concentrations. Morphologically, the Caco-2 and SW48 cells treated with low-concentration δT3 + Dox exhibited apoptotic features and great cellular stress. This combined treatment increased apoptosis rate, DNA damages (single and double stranded breaks) and enhanced DNA fragmentation. Mitochondrial membrane permeabilization (MMP) was observed on Caco-2 and SW48 cells treated with δT3 + Dox. The inhibition of MMP using cyclosporine a successfully suppressed cell death suggesting that the combined treatment targets at mitochondria. Despite activations of caspase-8 and -3 were detected, the application of caspase inhibitors was unable to improve cell viability signifying the possibility of caspase-independent cell death occurrence. Besides, the combined treatment induced an enhanced autophagy in the treated cancer cells as evidenced by the

formation of acidic vacuolar organelles as well as increments of monodansylcadaverine intensity and microtubule-associated protein 1A/1B light chain 3 (LC3)-II expression. The involvement of autophagic cell death is further confirmed when the application of autophagy inhibitors, namely 3-methyladenine and bafilomycin A1 successfully reverted cell death. Overall, current findings supported that δT3 + Dox combined treatment exerted both apoptosis and autophagy on Caco-2 and SW48 cells, where these multitargeted actions are highly imperative for treating the invasive type of colorectal cancer in the future.

P2066/B310

Inducible Degron Dependent Depletion of the RNA Polymerase I Associated Factor PAF53 Demonstrates It Is Essential for Cell Proliferation and Allows for the an alysis of Functional Domains.

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The regulation of ribosome biogenesis (RB) plays a central role in maintaining cellular homeostasis and supporting cell growth. The rate-limiting step in this process is transcription of the ribosomal RNA genes by RNA polymerase I (Pol I). Dysregulation of RB can contribute to pathologies such as cancer, cardiac hypertrophy, and ribosomopathies. Further, many chemotherapeutic drugs inhibit either rDNA transcription or rRNA processing, but have many off-target effects that limit their usefulness. Many pathways play a role in the regulation of rDNA transcription. Two mammalian factors that are involved in this regulation are **P**olymerase **A**ssociated **F**actor (PAF53) and PAF49. The purpose of this study is to determine the role(s) the PAFs play in regulating rDNA transcription and characterize the downstream physiological effects of directly inhibiting this process. To rapidly degrade PAF49/53, a novel system that utilizes CRISPR/Cas 9 and an auxin inducible degron was used. This allowed us to carry out in *vitro* biochemical and "genetic" studies of PAF49/53 in mammalian cells. Through the use of this system, our data show that PAF53 is required for rDNA transcription and cell proliferation. In addition, the three domains of PAF53 are each necessary but not sufficient to support wild-type levels of cell growth. The C-terminal tandem winged helix domain was only responsible for 20-30% of PAF53 function, while the linker region in combination with the N-terminal heterodimerization domain was able rescue cell proliferation by approximately 80%. Our lab has also defined a second DNA-binding domain in PAF53 that had not been discovered. Moreover, we have found a putative helix-turn-helix motif within this domain that is responsible for the DNA-binding activity. These findings are significant because they aid in further understanding the process of rDNA transcription by Pol I and the physiological consequences of inhibiting this process, *i.e.* nucleolar stress and cell arrest and/or death. They will also contribute to the discovery of novel drug targets that could be utilized in effective cancer and ribosomopathy treatments.

P2067/B311

CDK7 Inhibitor BS-181 Induces Extrinsic Apoptosis Via TRAIL/DR5 Upregulation That Is Preferentially Provoked in G_1-arrested Cells in Human Acute Leukemia Jurkat Cells.

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Chemotherapy resistance in human T-cell acute lymphoblastic leukemia (T-ALL), an aggressive neoplasm, results in poor prognosis despite advances in treatment modalities. We found that treatment of T-ALL Jurkat clone (JT/Neo) with the CDK7 inhibitor BS-181 induced extrinsic apoptosis via TNF-related apoptosis-inducing ligand (TRAIL)/death receptor 5 (DR5) upregulation, BID cleavage, BAK activation, mitochondrial membrane potential loss, caspase-8/caspase-9/caspase-3 activation, and PARP cleavage. BCL-2-overexpression (JT/BCL-2) abrogated these BS-181-induced apoptotic events, except for DR5 upregulation. Although CDK7-mediated activating phosphorylation of CDK1 (Thr-161) and CDK2 (Thr-160), and CDK1/2- and CDK4/6-preferred retinoblastoma phosphorylation at Thr-821/826 and Ser-795, respectively, were attenuated in BS-181-treated JT/Neo and JT/BCL-2 cells, only the latter exhibited G_1-arrest. The late G_1-blocking agent hydroxyurea augmented BS-181-induced apoptosis and BS-181-induced FITC-Annexin positive apoptotic cells represented mostly those in sub-G_1 and G_1 phases, indicating preferential BS-181-induced apoptosis in G_1-arrested cells. Exogenously added recombinant TRAIL (rTRAIL) synergized BS-181-induced apoptosis only in Jurkat clone A3 (wild-type) but not in I2.1 (FADD-deficient) and I9.2 (caspase-8-deficient) clones. The synergistic effect of rTRAIL on BS-181 cytotoxicity was not observed in normal peripheral T cells. These results demonstrated that BS-181 antitumor activity is mainly induced by extrinsic TRAIL/DR5-dependent apoptosis, and that BS-181 and rTRAIL in combination may hold promise for T-ALL treatment.

P2068/B312

Regulation of Expression of Oct3/4 by Farnesoid X Receptor and Liver X Receptors in Renal Cells and Feedback Regulation of the Expression of Oct3/4 and Its Upstream Factor Elavl2 in Renal Adenocarcinoma Cells.

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In the present study, we have found that nuclear receptors FXR and LXRs, originally characterized as regulatory factor which is involved in cholesterol/bile acid homeostasis, regulates the expression of Oct3/4, a marker for cell differentiation, in normal renal cell-derived cell line HK-2 and renal adenocarcinoma cell line ACHN. Down-regulation of Oct3/4 expression by activating FXR and LXRs occurred only in normal renal cell-derived HK-2 cells. Inverse agonist for LXRs-induced down-regulation of Oct3/4 in renal adenocarcinoma ACHN cells is a transient event arising from the loop regulation between Oct3/4 and its upstream factor ELAVL2. Given that mutation of the ELAVL2 structure possibly causes the loop regulation between Oct3/4 and ELAVL2, repairment of the ELAVL2 mutation is useful for the cancer therapy since it brings to cancel the system for keeping undifferentiated state of renal adenocarcinoma cells. Moreover, we revealed that LXR alpha and LXR beta regulate their expression each other. Although LXR beta-specific agonist is assumed to be the seeds for anti-arteriosclerotic drug only stimulating reverse cholesterol transport, it is needed that reveal the complex mechanism of regulation of LXR alpha and LXR beta for the development of such the anti-arteriosclerotic drug.

P2069/B313

Novel Post-translational Modification of Pml Sensitizes Sulforaphane (sfn)-induced Cell Cytotoxicity.

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Sulforaphane (SFN) is a natural compound and a promising therapeutic that induces apoptosis in cancer cells and inhibits cancer cell proliferation and tumorigenesis in various mouse models of cancer. However, our understanding of the mechanism by which SFN achieves its anti-tumorigenic activity is far from complete, in part, due to the lack of identification of SFN direct targets. We show that SFN redistributes the subcellular localization of the promyelocytic leukemia protein (PML), a well-established tumor suppressor, and inhibits the proliferation of MCF-7 and MDA-MB-231 breast cancer cells, and MCF-10A breast epithelial cells. PML is a stress sensor and an essential constituent of the highly dynamic PML nuclear bodies (NBs). Unexpectedly, loss of *PML* inhibits proliferation and migration of MCF-7 cells, and sensitizes the ability of SFN-mediated inhibition of cell proliferation, migration, and invasion. Consistently, overexpression of PML1, the most abundant *PML* spiced isoform in MCF-7 cells, increases cell proliferation, migration, and invasion. Mechanistically, SFN modifies several cysteine residues in PML, including C204, and impairs nuclear localization and NB formation. As a result, the mutant C204A is defective in nuclear localization, NB formation, and the colocalization with Daxx, Sumo1, and Sumo2/3, and is defective in its ability to inhibit proliferation, migration, and invasion of MCF-7 breast cancer cells. Together, our data reveal a novel mechanism in which SFN inactivates PML to elicit its cytotoxic activity.

P2070/B314

Cancer “Cross-talk” with Endothelial Cells Results in PSMA Expression.

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Angiogenesis provides nutrients and oxygen to cancer cells allowing them to flourish and often metastasize. If blood vessel recruitment and angiogenesis can be prevented, the cancer’s growth and spread might well be limited. The endothelial cells lining blood vessels vascularizing tumors overexpress an integrated transmembrane protein, Prostate-Specific Membrane antigen (PSMA), whose function has not been completely elucidated. The goal of this study was to elucidate why PSMA was expressed on tumor vasculature and not on normal vasculature. Our hypothesis is that tumor cells secrete factors and interact with endothelial cells within blood vessels resulting in PSMA expression. This study explored the effects of different cancer media on the expression of PSMA on human umbilical vein endothelial cells (HUVECs). The media were collected from a variety of cancer cell lines including prostate cancer C4-2 (PSMA+), prostate cancer PC-3 (PSMA-) and triple-negative breast cancer (MDA-MB 231). All experiments were done using growth-factor containing Matrigel on which cells were plated. Controls included HUVECs growing without cancer-conditioned medium, or not on growth-factor containing Matrigel. Targeted Molecular Imaging Agents (TMIAs), which bind to the PSMA receptor identified PSMA positive cells using confocal microscopy. The results demonstrated PSMA is induced in HUVEC cells growing in conditioned medium from each cell line; however the induction of PSMA was greatest with breast cancer cell conditioned medium. These studies should help elucidate the role of PSMA in angiogenesis and determine the factors involved in induction of the PSMA receptor on HUVECs by different cancer media. Targeting blood vessels vascularizing tumors and expressing PSMA may provide a method to limit or eradicate angiogenesis and thus limit the growth of the tumor.

P2071/B315

Investigation of the Cytotoxic Effects of Pro-apoptotic and Tumor Selective Piperidones.

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In vitro screening of compound libraries has led to the discovery of piperidones with significant potential as anti-cancer drugs. Piperidones, P3, P4, and P5, have potent cytotoxic activity and selectively kill cancer cells. In addition, we observed that these compounds induce cell death through apoptosis in leukemia HL-60 cells. This was revealed by significant phosphatidylserine externalization and activation of the executioner caspase, caspase-3. The intrinsic pathway of apoptosis was implicated in the mechanism of action of these compounds as shown by depolarization of the mitochondria and generation of reactive oxygen species. An alysis of the cell cycle profile revealed that these compounds also cause DNA fragmentation. Additionally, compounds P4 and P5 cause arrest at the G2/M phase. Finally, we found that these compounds and other similar compounds induce apoptotic activity by proteasome inhibition as shown by accumulation of poly-ubiquitinated proteins after compound treatment. Our results display a tumor selective cytotoxic effect that is mediated by both cell cycle and protein degradation mechanisms.

P2072/B316

Cervical Cancer Cells That Have Acquired Fgf13-Dependent Cisplatin-resistance Are Selectively Killed by Histamine Receptor H1 an tagonists.

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[Objectives] HeLa cisR cells, established from HeLa S cells by culturing them in cisplatin-containing medium, have autonomously acquired resistance to cisplatin and other platinum anticancer drugs. The strongly upregulated expression of fibroblast growth factor 13 (FGF13/FHF2) gene and protein in HeLa cisR cells was responsible for cisplatin resistance (Okada et al., Sci Rep. 2013; 3:2899). Thus, FGF13 appears to be a promising research target in order to overcome cisplatin resistance. Here we aimed at identifying compounds that are able to exert cytotoxic effects on HeLa cisR cells, thereby understanding the mechanisms by which FGF13 confers cisplatin resistance. [Methods] HeLa cisR cells and HeLa S cells were tested for their proliferation potential in the presence of various compounds. Compounds selected by this screening were examined in detail for their cytotoxicity and effects on cellular gene expression. [Results] Cloperastine, an approved antitussive drug with histamine receptor H1 antagonist activity, selectively kills cisplatin resistant HeLa cisR cells. Cloperastine was less cytotoxic toward cisplatin-sensitive parental HeLa S cells. Interestingly, FGF13-knocked-down line of HeLa cisR cells, that are cisplatin sensitive, could survive in the presence of Cloperastine at concentrations cytotoxic for HeLa cisR cells. Furthermore, Desloratadine and Clemastine, other histamine receptor H1 antagonists, demonstrate similar effects as Cloperastine. Other histamine receptor antagonists, i.e., H2 antagonist Nizatidine, H3 antagonist Pipolisant and H4 antagonist JNJ-7777120, exerted no or weak cytotoxicity to HeLa cisR cells. These results prompted us to examine the combined effects of cisplatin and Cloperastine on the mixed culture of HeLa S and HeLa cisR cells, and we found that this treatment killed both of these cells. [Conclusions and Discussion] HeLa cisR cells, the cervical cancer cells that have acquired cisplatin-resistance in an FGF13-dependent manner, are specifically killed by Cloperastine and other histamine

receptor H1 antagonists. The present findings will not only help us identifying the molecular mechanism involved in the FGF13-dependent cisplatin-resistance, but also will be practically valuable in treating cancers in which cisplatin-sensitive and cisplatin-resistant cells co-exist.

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Apoptotic Effect of Lambertianic Acid Via Suppression of Signal Transducer and Activator of Transcription 3 Phosphorylation and P65 Acetylation Mediated by Microrna134.

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Apoptotic effect of lambertianic acid via suppression of signal transducer and activator of transcription 3 phosphorylation and p65 acetylation mediated by microRNA134 Deok Yong Sim[$], Ji Hoon Jung[$], Hyo-Jung Lee, Eunji Im, Jisung Hwang and Sung-Hoon Kim [$] These authors contribute equally to the work. As p300-mediated RelA/p65 hyperacetylation by signal transducers and activators of transcription 3 (STAT3) is critical for NF-κB activation, in the current study, the apoptotic mechanism of lambertianic acid (LA) was explored in relation to STAT3 phosphorylation and RelA/p65 acetylation in MCF-7, DU145, PC-3, and MDA-MB-453 cells. LA significantly increased the cytotoxicity, sub G 1 population, and the cleavage of poly (ADP-ribose) polymerase (PARP) in MDA-MB-453 or PC-3 cells (STAT3 mutant), more than in the MCF-7 or DU145 cells (STAT3 wild). Consistently, LA inhibited the phosphorylation of STAT3 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and disrupted the interaction between p-STAT3, p300, NF-κB, and RelA/p65 acetylation (Ac-RelA/p65) in the MCF-7 and DU145 cells. Also, LA reduced the nuclear translocation of STAT3 and NF-κB via their colocalization, and also suppressed the protein expression of XIAP, survivin, Bcl-2, Bcl-xL, vascular endothelial growth factor (VEGF), Cox-2, c-Myc and mRNA expression of interleukin 6 (IL-6), and tumor necrosis factor-α (TNF-α) in MCF-7 cells. Conversely, IL-6 blocked the ability of LA to suppress the cytotoxicity and PARP cleavage, while the depletion of STAT3 or p300 enhanced the PARP cleavage of LA in the MCF-7 cells. Notably, LA upregulated the level of miRNA134 and so miRNA134 mimic attenuated the expression of pro-PARP, p-STAT3, and Ac-RelA, while the miRNA134 inhibitor reversed the ability of LA to reduce the expression of Ac-RelA and pro-PARP in MCF-7 cells. Overall, these findings suggest that LA induced apoptosis via the miRNA-134 mediated inhibition of STAT3 and RelA/p65 acetylation.

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Efficacy Assessment and Mechanism Exploration of Kruppel Like Factor 2 Targeting Intervention Strategy for Non Small Cell Lung Cancer Therapy.

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Non-small cell lung cancer (NSCLC), the most common type of lung cancer, has the problems such as less sensitivity to chemotherapy and resistance to tyrosine kinase inhibitors. Kruppel-like factor 2 (KLF2) has been reported to have the potential activities in tumor suppression and immune regulation. The expression level of KLF2 is lower in NSCLC and negatively correlated with the stages of tumor progression and lymph node metastasis. To date, not only no KLF2-promoting compounds have been developed, but no related studies on its anti-tumor efficacy in vivo have been verified. Therefore, the purposes of this study were focused on the potential and related mechanisms of KLF2 applied as a targeted molecule for NSCLC therapy. Our data indicated that the intervention of lovastatin markedly up-regulated protein expression of KLF2 in A549 human non-small cell lung cancer cells. Moreover, KLF2 siRNA intervention could significant inhibit serum-induced cell growth in A549 cells. The analysis result

of the Ingenuity pathway analysis (IPA) showed that several intracellular molecules, such as AKT, mTOR and FOXO1, maybe the potential upstream regulators to involve in KLF2 expression. Besides, the experimental data also evidenced that lovastatin can suppress activations of AKT and mTOR proteins as well as to reduce protein expression of FOXO1. In summary, these results suggested that intensifying KLF2 expression could apply as a feasible therapeutic strategy to improve NSCLC therapy, and statinoid compounds have potential to be developed as KLF2 activator. In addition, activations or expressions of AKT-mTOR pathway and FOXO1 may be attributed to suppression of KLF2 in NSCLC cells, such as A549 cells.

P2075/B319

Cytotoxic Effects of Ajoene on Adherent and Non-adherent Cancer Cell Lines.

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Ajoene Abstract Garlic is a common ingredient with a long history of human use both as a food and a medicine. When garlic is processed, allicin molecules are released and a chemical reaction occurs forming ajoene, an organosulfur compound. Previous studies have shown ajoene to have anti-cancerous properties such as inhibiting cell proliferation, inducing apoptosis, and reducing adhesion in solid tumor cancer cells. Based on these previous findings, we hypothesize that treating non-adherent (leukemia and myeloma: Jurkat, RPMI 8226, and CML) and adherent (prostate and breast: LNCAP and MCF7) cancer cell lines with ajoene will result in lower viability due to increased apoptosis induced by the compound. In this study, these cells were plated and exposed to varying concentrations of ajoene ranging from 20 to 120 μM and assessed for viability, cytotoxicity and apoptosis. Our findings indicate that cell viability was statistically reduced in a dose responsive manner in all non-adherent cell lines when exposed at the lower concentrations of 20 to 80 μM ajoene exposure, while the adherent cell lines required a higher exposure of 120 μM. In order to begin to elucidate the manner in which cell death was being mediated by ajoene exposure, we exposed cancer cells to 120 μM ajoene and monitored them over a 48 hr period using a biochemical assay that distinguishes between apoptosis and necrosis. These results show that ajoene exposure induces apoptosis initially and then it is followed by secondary necrosis in all studied cell lines. Moreover, with the exception of the CML line, ajoene appears to cause greater amounts of cell death via these pathways than positive control cisplatin. Currently real time-PCR and western blots are being performed to further support the cell death mechanistic findings.

P2076/B320

Vitamin D Can Enhance the Effects of Anti-tumor Treatment in Prostate Tumor Cells.

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The incidence rate of prostate cancer has been rapidly increased since the mid-1990s and is thought that due to the improvement of detection sensitivity using the PSA test, longevity medicine, changing the dietary habit, and so on. Recent cohort studies regarding vitamin D for cancer prevention have shown that vitamin D concentration in plasma was inversely associated with the risk of multiple cancers, but beneficial effect on prostate cancer progression is under dispute among the researchers. To dissect this issue at the molecular and cellular level, we investigated the molecular basis and cellular response of prostate cancer cell triggered by VD3 treatment. In this study, we identified that IGFBP3 induced by VD3-VDR axis was responsible for the suppression of cell growth and IGFBP3 improved the effect of

docetaxel, one of the first-line agents for prostate tumor treatment, in LNCaP cells. We also found that Bcl-2 protein expression level was down-regulated by VD3 treatment in an IGFBP3 independent manner. These data suggested that VD3 treatment could positively contribute to prostate tumor treatment. Additionally, we also found that transient VD3 treatment-induced prolonged IGFBP3 expression and IGFBP3 induction level seemed to be adjusted by checking protein concentration, expecting the existence of positive-feedback loop for IGFBP3 retention. In conclusion, VD3 treatment in combination with active surveillance or chemotherapy would be helpful to improve the outcome of patients with prostate cancer.

P2077/B321

Morin Increases Pannexin 2 Expression and Induces Apoptosis in U87 and U251 Glioblastoma Cells.

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Glioblastoma multiforme (GBM) is the most common and aggressive malignant primary brain tumor in human. Pannexin 2 is a member of pannexin family that mainly presents in endomembrane system. Recent work suggests that pannexin 2 reduces cell proliferation and regulates apoptosis. Morin, a flavonoid originally isolated from figs and members of the moracea family, has previously shown to possess anti-tumor activities and induce apoptosis in several human cancer cell lines. In this study we examined the cytotoxic effects of morin in the U87 and U251 human glioblastoma cells by MTT assay and the ability of morin inhibiting single cells to grow into colonies by colony formation assay. We also evaluated anticancer potential of morin (300 μM) through induction of apoptosis in U87 and U251 cells by Western blot and annexin V-FITC fluorescence microscopic analyses. Morin reduced both the viability and the colony formation ability of U87 and U251 cells in a dose- and time-dependent manner. An nexin V-FITC fluorescence microscopy showed that morin induced apoptosis in U87 and U251 cells. Morin treatment also increased the expression levels of cleaved caspase-3 and pannexin 2 in U87 and U251 cells as demonstrated by Western blot and immunofluorescence microscopic analyses. Moreover, blocking the activation of caspase-3 by caspase inhibitor z-DEVD-FMK inhibited the morin-induced increase in pannexin 2 levels in U251 cells. These results suggest that morin may have potent anticancer activities against U87 and U251 cells via upregulating pannexin 2 to cause apoptosis.

P2078/B322

The Nicotinic Acetylcholine Receptor Modulator 4R as a Treatment for Non-small Cell Lung Carcinoma.

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Introduction: **Lung cancer** is the leading cause of cancer deaths worldwide, with 85% of new cases classified as non-small cell lung carcinoma (NSCLC) and a patient survival rate <5%. While most treatments remain cytotoxic-platinum based, novel non-toxic compounds like the 4R cembranoid have shown antiangiogenic, anticancer and antitumorigenic activity. We **aim** to study the potential of 4R against NSCLC via the modulation of nicotinic acetylcholine receptors (nAChRs) and **hypothesize** 4R will decrease cancer progression by inhibiting the α7nAChRs signaling in lung tumor and endothelial cells. *Methods:* 4R effect on tumor growth in *vivo* was evaluated using the Lewis Lung Carcinoma (LLC) mouse model; C57BL/6J mice were monitored for 12 days and tumors were evaluated for volume and angiogenesis markers (Western Blot). NSCLC cells (LLC and A549) were evaluated for: α7nAChR expression (Flow Cytometry and Western Blot), cell viability after 4R exposure (MTT assay or propidium

iodide staining), proliferation and apoptosis markers (Western Blot). *Results:* Treatment with 4R significantly reduce tumor size in *vivo*, while (1) no significant difference was observed in PECAM-1 (pro-angiogenic marker) expression; and (2) no effect on tumor cell viability was observed in *vitro*. Both NSCLC cells, show α7nAChR expression, while 4R treatment of A549 seemed to affect cell viability and deregulate (proliferation and apoptotic) protein expression. *Conclusions:* 4R significant tumor reduction suggests this drug to be a potential non-toxic treatment or adjuvant therapy against NSCLC. Although the mechanism of action is yet to be elucidated, it does not appear to involve a decrease in angiogenesis or a direct effect on tumor cells' viability. Experiments to assess the effect of 4R in NSCLC cells migration and invasion are currently ongoing. *Acknowledgments:* This work is supported by the NIH NINDS SNRP grant number 5U54NS083924-03, the NIH NIMHD and the NIH NIAID grant number U54MD007587, and the PR-INBRE program Supported by an Institutional Development Award (IDeA) from the NIH NIGMS grant number 5P20GM103475-17. Approved by IACUC.

P2079/B323

Understanding the Underlying Mechanisms of Cell Death in Mitotically Arrested Cancer Cells.

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Anti-mitotic drugs represent a common strategy for cancer chemotherapy by inducing prolonged prometaphase delay and cell death. However, studies both in vitro and in xenograft tumor models indicate that cancer cells display a high degree of heterogeneity in their responses to mitotic arrest, from dying during mitosis to surviving mitotic failure and continuing to progress through the cell cycle. Thus, understanding the basis for this variation may prove valuable for developing more effective anti-mitotic chemotherapeutic strategies. Based on reports suggesting that signaling pathways that regulate survival might account for a cell's ability to survive mitotic arrest, we asked whether inhibition of the PI3-kinase pathway affected the kinetics of apoptosis in cells exposed to a KSP inhibitor. Simultaneous inhibition of KSP and PI3K/AKT induced apoptosis more effectively than mitotic arrest or PI3K/AKT inhibitors alone, and tracking of individual cell fates revealed that cells subjected to the combinatorial treatment died significantly sooner than cells subjected to KSP inhibition alone. To determine whether these cells died during mitosis or during mitotic slippage, we employed a membrane-tagged caspase biosensor co-expressed with fluorescent tubulin or cyclin B, which enabled us to determine cell cycle status during which cells died in response to mitotic delay. In our hands, HeLa cells typically died during mitotic slippage, and tracking of cells expressing these biosensors displayed a narrow range of cellular responses, from apoptosis during mitotic arrest to mitotic slippage and cell survival. However, we found that mitotic arrest in combination with PI3K inhibition dramatically shifted the dynamics of cell death from apoptosis during mitotic slippage to apoptosis during mitotic arrest. Thus, the acceleration of cell death induced by PI3K inhibition was due to a shift in when cells died relative to mitotic progression, rather than an acceleration of the kinetics of mitotic slippage. Together, these results not only shed insights into the possible reasons to why cells respond differently to mitotic delay, but also suggest a strategy whereby anti-mitotic interventions may be optimized.

P2080/B324

Localization of Porphyrin TMP in B16F1 Mouse Melanoma Cells and Its Potential Use in Photodynamic Therapy.

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Photodynamic therapy (PDT) is a treatment that uses photosensitizing agents and specific wavelengths of light to kill cancer cells. The photosensitizing agents produce reactive oxygen species when exposed to light, which induce cell death when the reactive oxygen species bind to target sites. The effectiveness of PDT depends on the localization of the photosensitizing drug inside cancer cells. The ideal drug delivery should enable the selective accumulation of the photosensitizing drug within the diseased tissue and the delivery of therapeutic concentrations of drugs to the target site, with little uptake by healthy cells. Photosensitizing porphyrins, whose molecules contain a flat ring of four linked heterocyclic groups, are a typical choice for the photosensitizing agents. In this study we tested a novel porphyrin called TMPyP4 (TMP) using fluorescent organelle probes to determine its subcellular localization in B16F1 mouse melanoma cells. We discovered that TMP localizes to the endoplasmic reticulum in cells treated with 3.0 μg/mL over a 1-hour period. Future studies will examine TMP localization in other organelles, and test the effectiveness of TMP in causing light-stimulated cell death in B16F1 cells.

P2081/B325

Stat3 Contributes to Thermotolerance through Induction of Hsp105 in Mammalian Cells.

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Themotolerance is one of the mechanisms that maintain homeostasis of mammalian cells. Pretreatment with a non-lethal heat shock induces thermotolerance, which causes cell resistance against subsequent lethal heat shock. Thermotolerance is associated with the synthesis and cellular accumulation of heat shock proteins (Hsps), including Hsp70 and Hsp27. We previously showed that the expression of Hsp70 is regulated by the cytokine signaling transcription factor Stat3. However, the role of Stat3 in thermotolerance is not known. In this study, we examined the possibility that Stat3 regulates thermotolerance development. Western blot analysis using anti-phospho-Stat3 (Tyr705)-specific antibody revealed that non-lethal mild heat shock induces the Stat3 phosphorylation in HeLa cells and HepG2 cells. The heat shock-induced Stat3 phosphorylation was inhibited by the JAK tyrosine kinase inhibitor AG490. Furthermore, AG490 partially suppressed the mild heat shock-induced thermotolerance and the induction of Hsp105, Hsp70, and Hsp27, suggesting that heat shock stimulates the JAK-Stat signaling pathway for development of thermotolerance. Mild heat shock-induced phosphorylation, Hsps induction, and thermotolerance were suppressed by the Stat3 inhibitor Stattic, confirming that Stat3 is involved in thermotolerance development. We further demonstrated that the knockdown of Hsp105 decreases the mild heat shock-induced thermotolerance using HeLa cells stably expressing Hsp105-specific shRNA or non-targeting shRNA. These findings indicate that the activation of Stat3 signaling pathway is required for the development of thermotolerance through the induction of several Hsps including Hsp105. The acquisition of thermotolerance is a problem of hyperthermia treatment. These results suggest that Stat3 inhibition may be useful target for hyperthermia cancer therapy.

P2082/B326

RNAi-mediated Downregulation of LAT1/SLC7A5 Expression and Function in Human Colon Cancer Cells.

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Overexpression of L-type amino acid transporter 1 (LAT1/SLC7A5) helps support the continuous growth and proliferation of cancer cells through mTOR activation and has been suggested to be a marker of colorectal cancer prognosis and metastatic potential (Cancer Sci*99*: 2380-2386, 2008; anticancer Res 30: 4223-4227,2010; Oncol Lett 14: 7410-7416, 2017). The present study examined the effect of *LAT1* gene downregulation by RNA interference on the mRNA and protein expression, on the cellular function of the transporter and cell proliferation of human colon cancer HCT-116 cells. siRNAs targeting human *LAT1* sequences PB-8603-111t (Phyzat), SI31011000 (Qiagen) and HSS112005 (Invitrogen) and negative control siRNA commercial sequences NC-SI03650318 and NC-SI03650325 (Qiagen) were tested. HCT-116 cells were transfected with 25 nM siRNAs complexed with Lipofectamine 2000® or Injectin®, and their effects upon LAT1 mRNA expression (RT-qPCR), protein abundance (western blot), inward transport of [^{14}C]-L-leucine and cell proliferation (calcein fluorescence) were evaluated after 72 h. [^{14}C]-L-leucine accumulation in HCT-116 cells was reduced in the presence of the LAT1 inhibitor BCH in a concentration-dependent manner. LAT1 mRNA expression was reduced by 47%, 62% and 73% after treatment with SI31011000, HSS112005 and PB-8603-111t siRNA sequences, respectively, while LAT1 protein expression was reduced to around 50%. This effect was accompanied with a reduction of [^{14}C]-L-leucine accumulation in HCT-116 cells. Moreover, LAT1 downregulation decreased cell proliferation, particularly when treated with PB-8603-111t. The negative control siRNAs were devoid of effects on the parameters measured. Importantly, intra-tumoral injections of PB-8603-111t caused a decrease in relative tumor volume in immune deficient mice injected subcutaneously with HCT-116 cells. In conclusion, we expect the anti-LAT1 siRNA PB-8603-111t to decrease tumor growth and metastasis potential in human colon cancer. Part of this work was developed within the SIRNAC project, co-financed by NORTE2020, PT2020 and the European Union.

P2083/B327

RNAi-mediated Downregulationof ASCT2/SLC1A5 Expression and Function in Human Colon Cancer Cells.

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Overexpression of alanine/serine/cysteine transporter 2 (ASCT2/SLC1A5) helps support the continuous growth and proliferation ofcancer cells through mTOR activation and has been suggested to be a marker of colorectal adenocarcinoma prognosis (Anticancer Res 22:2555-2557,2002; Mol Imaging Biol 19:421-428,2017). The present study examined the effect of *ASCT2* gene downregulation by RNA interference on the mRNA and protein expression, transport function and proliferation of human colon cancer HCT-116 cells. siRNAs targeting human ASCT2 sequences PB-4501-134t (Phyzat) and SI00079730 (Qiagen) and negative control siRNA commercial sequences NC-SI03650318 and NC-SI03650325 (Qiagen) were tested. HCT-116 cells were transfected with 25 nM siRNAs complexed with Lipofectamine 2000® or

Injectin®, and their effects upon ASCT2 mRNA expression (RT-qPCR), protein abundance (western blot), inward transport of [^{14}C]-L-alanine and cell proliferation (calcein fluorescence) were evaluated after 72 h. Anti-ASCT2 siRNAs reduced ASCT2 mRNA expression in HCT-116 cells by 70% and 74% and ASCT2 protein expression by 44% and 63% with SI00079730 and PB-4501-134t, respectively. This effect was accompanied by a reduction of intracellular of [^{14}C]-L-alanine accumulation (by 23% and 49% with SI00079730 and PB-4501-134t, respectively). Moreover, ASCT2 downregulation decreased cell proliferation, particularly when treated with PB-4501-134t (by 78%). Importantly, intra-tumoral injections of PB-4501-134t caused a decrease in relative tumor volume in immune deficient mice injected subcutaneously with HCT-116 cells. In conclusion, we expect the anti-ASCT2 siRNA PB-4501-134t to decrease tumor growth and metastasis potential in human colon cancer. Part of this work was developed within the SIRNAC project, co-financed by NORTE2020, PT2020 and the European Union.

P2084/B328

Effects of Acid Ceramidase Over-Expression on H295R Adrenal Carcinoma Cells.

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The enzyme acid ceramidase has been shown to be over-expressed in some cancer cell lines and primary tumors, and enhances the ability of these cells to convert ceramide, which is often produced as a pro-apoptotic response to stress, to sphingosine, which can then be converted to the pro-survival molecule sphingosine-1-phosphate. Therefore, acid ceramidase over-expression confers a survival advantage in response to stress such as chemotherapy and radiation. Cortisol is a steroid stress hormone produced by adrenal cells in response to adrenocorticotropic hormone released by the pituitary gland. Published studies have shown that adrenocorticotropic hormone can also increase acid ceramidase expression in these cells. In order to evaluate the effects of increased acid ceramidase expression on adrenal cells, H295R adrenal carcinoma cells were engineered to have consistent high-level acid ceramidase expression. These cells were evaluated for ceramide resistance, cortisol production, and receptor expression.

P2085/B329

NAG-1/GDF-15 Expression in Thyroid Cancer Development•

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Thyroid cancer is one of the most common cancer in worldwide and incidence of this cancer has been increased during the last decades. In addition, unnecessary surgeries are being carried out due to no specific biomarker for the diagnose. Therefore, identification of novel biomarkers should be considered for thyroid cancer diagnosis. Antibody arrays were performed using normal and tumor tissue of thyroid cancer patient and found several biomarkers that could be used in thyroid cancer patients. Among the candidate proteins chosen based on the antibody array, NAG-1/GDF15 exhibited increased expression in normal tissues, compared to adjacent tumor tissues. Since NAG-1 is secreted protein, ELISA assay was performed using plasma of thyroid cancer patients to measure concentration of NAG-1/GDF15. NAG-1 levels increased with age as consistent with previous reports, but there was no significant difference in thyroid cancer type, sex, and BMI. Interestingly, NAG-1 expression was increased when phytochemicals and NSAIDs were treated into thyroid cancer cell line BcPAP, with dose-dependent manner. We further showed that combination treatment of lenvatinib with sulindac sulfide, or quercetin showed NAG-

1/GDF15 induction and synergistic effect on anti-cancer activity, measured by CX7 High-Content Screening (HCS) Platform. Finally, western blot analysis was performed using tissues from follicular thyroid cancer patients, and NAG-1/GDF15 expression was able to distinguish follicular adenoma (FA), follicular variant of papillary thyroid cancer (FVPTC), and follicular thyroid carcinoma (FTC). Overall, our study indicated that NAG-1 may provide a novel biomarker for the thyroid cancer prognosis and even differentiate different thyroid cancer types.

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Epigenetics and Chromatin Remodeling

P2086/B331

Inositol Kinase Activity of Ipmk Is Essential for Activation of ClassI Hdac.

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IPMK (Inositol polyphosphate multikinase) is the rate limiting enzyme in inositolphosphate biosynthetic pathway. IPMK catalyze production of inositoltetraphosphate (IP4) and inositol penta phosphate (IP5), which is conserved in all the species. In mammal, IPMK can also function as a PI3Kinase. By tandem affinity purification (TAP) followed by massspectrometric analysis we found HDAC1 as an interacting partner of IPMK. Further validation by over expression and endogenous immunoprecipitation study proved the TAP data. Confocal imaging also showed IPMK/HDAC1 colocalization. Interestingly deletion of IPMK in MEF cells abolished HDAC1, HDAC2 and HDAC3 activity but made no impact on HDAC8. Interestingly, over expression of wild type IPMK can rescue HDAC activity in IPMK deleted MEFs, failed by kinase dead form of IPMK. Arabidopsis IPMK which has IP4/IP5 kinase activity but devoid of PI3Kinase activity can rescue the HDAC activity as comparable to wild type. Thus, we confirm that inositol kinase activity of IPMK influences HDAC activity.

P2087/B332

Regulation of Chromatin Structure by *Drosophila* Nrf2-Keap1 Xenobiotic Response Factors.

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The Nrf2-Keap1 pathway regulates transcriptional response to xenobiotic and oxidative stress, thereby reducing the adverse effects of these compounds on the health of an organism. According to the classic model, Keap1 interacts with Nrf2 in the cytoplasm and targets Nrf2 for ubiquitination and degradation. When xenobiotic compounds or reactive oxygen species are present, they disrupt the interaction between Keap1 and Nrf2, releasing Nrf2 to enter the nucleus and activate transcription of response genes. Although the short-term responses to xenobiotic factors are well understood, the mechanisms that mediate the effects of long-term exposure to xenobiotics on development remain unknown. It has been found that the Nrf2-Keap1 pathway can regulate normal development in *Drosophila and* mice. For example, *Drosophila* Nrf2 and Keap1 (CncC and dKeap1) regulate the synthesis of and response to ecdysone (an important hormone in metamorphosis). Elucidating other mechanisms by which Nrf2 and Keap1 can regulate development could help us understand how xenobiotics affect development and the complicated roles of Nrf2 and Keap1 in disease. Given the importance of epigenetic regulation in development, we investigated the role of CncC and dKeap1 in regulating chromatin packaging. A position effect variegation (PEV) assay uses a transcriptional reporter gene to assess heterochromatic gene silencing. PEV assays revealed that knockdown of CncC and/or dKeap1 resulted in a reduction of

gene silencing caused by pericentric heterochromatin. Knockdown of CncC or dKeap1 in embryos reduced the level of heterochromatin marker histone H3K9me2. However, knockdown of CncC or dKeap1 did not affect the expression levels of genes that encode the heterochromatin components Su(var)3-9 and HP1. These results indicate that CncC and Keap1 promote or maintain pericentric heterochromatin formation, likely at the post-transcriptional level. Thus, epigenetic regulation of chromatin may represent a novel mechanism by which the Keap1-Nrf2 xenobiotic response signaling pathway controls development. Future studies will aim to determine the interacting partners of Nrf2 and Keap1 and establish whether Nrf2 and Keap1 also regulate euchromatin structure. Elucidating the roles of Nrf2 and Keap1 will help us understand how environmental toxins may impact epigenetics, development, and human health.

P2088/B333

The Interplay between Genomic Instability and Mitochondrial Homeostasis in the Brain.

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Phosphorylation of histone H2AX is a major contributor to efficient DNA repair. We recently reported neurobehavioral deficits in mice lacking H2AX. Here we establish that this neural failure stems from impairment of mitochondrial function and repression of the mitochondrial biogenesis gene PGC-1α. H2AX loss leads to reduced levels of the major subunits of the mitochondrial respiratory complexes in mouse embryonic fibroblasts and in the striatum, a brain region particularly vulnerable to mitochondrial damage. These defects are substantiated by disruption of the mitochondrial shape in H2AX mutant cells. Ectopic expression of PGC-1α restores mitochondrial oxidative phosphorylation complexes and mitigates cell death. H2AX knockout mice display increased neuronal death in the brain when challenged with 3-nitropronionic acid, which targets mitochondria. This study establishes a role for H2AX in mitochondrial homeostasis associated with neuroprotection.

P2089/B334

Epigenetic Alterations of Environmental Endocrine Disruptors Can Be Countered by Phytochemicals Resulting in Loss of Proliferation of the MCF7 Breast Cancer Cells.

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Breast cancer is a highly mortal disease in females with its incidence is estimated to increase by 58% by 2040 according to the Global cancer observatory of IARC. Among the other factors, environmental endocrine disruptors (EEDs) are known to switch on or off breast cancer-associated genes by altering epigenetic mechanisms. The objective of the present study is to investigate the anti-proliferative mechanisms of some phytochemicals resulting in a reversal of the epigenetic alterations due to exposure of such EEDs. Using the MCF7 breast cancer cells, the alterations in the expression of the epigenetic regulators of histone and DNA including the protein arginine methyltransferase 5 (PRMT5), histone deacetylase 1 (HDAC1), enhancer of zeste homolog 2 (EZH2) and DNA methyltransferase 1 (DNMT1) by exposure to EEDs and phytochemicals alone or in combinations was analyzed by qPCR and western blotting. The results showed the induced proliferation of the MCF7 cells by the EEDs was significantly reduced when the phytochemicals were present. The expression and function of the above epigenetic enzymes upregulated by the EEDs was reversed by the phytochemicals which result in significant global loss of the repressive histone methylation marks (H3K27me3 and H4R3me2s),

decreased enrichment of these marks along with demethylation of the CpG islands located within the promoters of p21, p53, and BRCA1 in the MCF7 cells within 24 hours. It was therefore hypothesized that the increased level of repressive epigenetic factors in the EED-exposed cells was reversed by phytochemicals (including brazilin, curcumin, epigallocatechin gallate, ellagic acid, and resveratrol) used in the study resulting in the restoration of the above anti-proliferative genes contributing to the loss of proliferation of the malignant MCF7 cells. In summary, the findings of the present study reveal novel epigenetic mechanisms critical for induced cell proliferation by such environmental chemicals could be reversed by the dietary phytochemicals with anticancer properties.

P2090/B335

Dynamic Interplay between De-novo DNA Methylation and Chromatin Structure during the Loss of Pluripotency.

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During early development exit from pluripotency and priming for differentiation into somatic lineages is associated with genome-wide de novo DNA methylation. As a result of these processes cells carry distinct epigenetic marks that are associated with their fate during later stages of development and adulthood. But how are these epigenetic marks robustly established? Combining novel methods from single-cell multi-genomics and theoretical biophysics we infer the mechanisms governing de-novo methylation via DNMT3 enzymes. We show that the establishment of DNA methylation is a collective process involving feedback ranging over large genomic domains. Surprisingly, de-novo methylation follows generic dynamics which are self-similar in time and space. We demonstrate how these epigenetic marks are established through the interplay between chemical and topological modifications of the DNA and show how such processes provide a proofreading mechanism for the establishment of methylation marks. Our work sheds new light on epigenetic mechanisms involved in cellular decision making. It also highlights how mechanistic insights into the molecular processes governing cell-fate decisions can be gained by the combination of methods from single-cell multi-genomics and theoretical biophysics.

P2091/B336

The Stag2-cohesin Functions in Brain Development.

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Mutations of the cohesin components and regulators result in developmental diseases with multisystem dysfunction in human, which collectively referred to as cohesinopathes. Recently, familiar germline mutations in the X-linked gene STAG2 were found in a novel type of cohesinopathy that features in intellectual deficiency. Cohesin is a ring-shaped protein complex that entraps and tethers DNA segments. Other than mediating sister-chromatid cohesion, it also promotes DNA damage repair, and regulates transcription. In this study, we created a brain-specific Stag2 conditional knockout mouse

model. We discovered that the cohesion subunit Stag2 plays an important role in the mouse brain development and myelination, through promoting transcription in oligodendrocytes. Stag2 CNS-specific knockout mice fail to thrive. The CKO were born at normal mendelian ratio. However, the CKO present growth retardation and premature death while the floxed control litter mates grow up normally. Till four weeks old, about half of the CKO died, with the rest CKO mice weighing only half of the control mice and dying within three months. Despite having lower plasma Igf-1 level, Stag2 CKO display normal feeding and drinking. Meanwhile, the Stag2 CKO pups present neurological defects: forepaw and hindlimb clasping, and the ataxia-like behavior. Using RNA sequencing, we further discovered that cholesterol biosynthesis and myelination was repressed during development in CKO. By Mass spectrometry, we found that all major cholesterol precursors are at lower levels in CKO. In *situ* hybridization confirmed that the myelin mRNA levels are reduced in P14 CKO. Our Electron Microscopy results also showed the myelination deficiency in CKO brains. Although, oligodendrocyte progenitor cells (OPCs) number counting in CKO is comparable to Ctrl. We found OPC differentiation in *vitro* displayed abnormalities. In *vitro* differentiated oligodendrocytes from Stag2 CKO have significant fewer branching process than the control. This suggests that Stag2 deficient oligodendrocytes are able to form but have impaired function in myelinating axons. Our study proved that a cohesin subunit plays important roles in the development of mammals by regulating transcription of a specific cell type.

P2092/B337

Partitioned Heritability and Functional Enrichment Reveal Ovarian Cancer Risk Variants in Histotype-specific Enhancers That Disrupt Transcription Factor Binding Sites.

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Genome wide association studies (GWAS) have identified 41 regions associated with risk of epithelial ovarian cancer (EOC). Given that the vast majority of these variants lie in the non-coding genome, and that regulatory landscapes are highly tissue specific, we hypothesized that EOC risk alleles disrupt regulatory regions and function through their interaction with epigenomic features in cell types relevant to EOC. We applied computational tools to identify functional mechanisms at GWAS risk loci for EOC. We first estimated the heritability explained by common SNPs for each EOC histotype and found that the current known risk loci of high grade serous ovarian cancer (HGSOC) account for approximately 6% of narrow sense heritability (h_g^2) Moreover, we partitioned SNP-heritability across non-cell-type-specific functional categories and observed a significant contribution of regulatory elements to EOC heritability, e.g. Promoter (p = 0.016), and Super-enhancer (p = 0.02). To understand how risk variants residing in regulatory elements affect cancer development through the epigenomic landscape, we collected epigenomic datasets for a total of 121 cell and tissue types, from public resources (Roadmap Epigenomics and ENCODE) and in-house generated ChIP-seq. We identified significant enrichment of EOC risk variants in cell and tissue specific active regulatory elements marked by H3K27Ac. Intriguingly, credible causal SNPs for HGSOC were enriched in H3K27Ac only in HGSOC tumors, and depleted in ovarian surface epithelium demonstrating that germline risk variants for EOC are located within regulatory elements that are active in tumor, rather than precursor normal tissues, but also add to the existing evidence that ovarian surface epithelium are unlikely the cell of origin for HGSOC. We further examined the effect of credible causal variants within H3K27Ac regions in EOC related cell types on

transcription factor binding sites. The most frequently broken motif was REST, which is disrupted by 19 SNPs and has been implicated as both a tumor suppressor and an oncogene. Overall, these systematic analyses guide comprehensive interpretation of EOC risk variants. Functional annotation with epigenomic data from relevant cell types can identify putative causal regulatory elements and transcription factor binding sites that are disrupted by risk variants for EOC.

P2093/B338

Actin Alignment Enhanced in Endothelial Cells Under Shear Stress Due to Decreased Nuclear Stiffness.

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Nuclear shape and architecture play an important role in how the cell responds to physiological forces. Aberrant structural changes in the nucleus are seen in several human diseases such as cancer, laminopathies, heart disease, and muscular dystrophy. We therefore sought to examine how changes in nuclear structure and chromatin condensation affect the endothelial cell (EC) response to shear stress. Human Umbilical Vein Endothelial Cells (HUVECs) were treated with histone deacetylase inhibitor, trichostatin a (TSA), to increase euchromatin, softening the nucleus, and a histone demethyltransferase inhibitor, methylstat, to increase heterochromatin, hardening the nucleus. In addition, we also used VEGF, previously shown to increase chromatin mobility, to soften the nucleus. HUVECs were exposed to both oscillatory and laminar shear stress, in the presence and absences of these modifications, and assayed for DNA damage and actin alignment in the direction of flow. We observed that both TSA- and VEGF-treated cells, had faster cell alignment under laminar shear stress than condensed DNA or wildtype. HUVECs treated with TSA and VEGF align parallel in the direction of flow after 24 hours, whereas the wildtype cells (untreated) only align after 72 hours. In contrast, no alignment was observed in the methylstat group at 72 hours, suggesting that increased chromatin condensation results in ECs that cannot properly adapt to shear stress. Significant phosphorylation of H2AX, a marker for DNA damage, was detected in cells treated with methylstat under laminar and oscillatory flow, suggesting that a stiffer nucleus is more prone to DNA damage. In conclusion, this data shows that changes in nuclear stiffness affects the ability of endothelial cells to adapt and regulate force.

P2094/B339

Simple Sequence Repeats -Deciphering Functions in the Genome.

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Simple Sequence Repeats (SSRs) or Microsatellites are short tandem repeats of 1-6 nucleotide motifs with their repetitive unit occurring anywhere between 10-20 times. SSRs occur in most organisms, in coding and non-coding DNA but their functions are unknown.In the human genome, they are twice as abundant as the protein-coding DNA. Studies suggest that SSRs may have essential roles in gene regulation and genome organisation. Recently, the disease associated SSRs have been shown to occur in CpG island rich chromatin boundaries and cause topological disruptions on expansion. Chromatin domains in the genome are defined by DNA elements called Insulators/boundary elements. The globin insulator, the *Drosophila* Gypsy insulator and the boundaries of *Drosophila Hox* loci are well-known insulators. Repeats can function as enhancer-blockers preventing communication between a promoter and an enhancer or as barrier elements protecting transgenes from position-dependent silencing. The

GATA-repeat is shown to have enhancer blocking activity in *Drosophila* and human cell lines. As interesting candidates for such function, we selected 23 human SSRs based on their 'length preference' to study their cis-regulatory potential. Using cell-based functional assays to study their cis-regulatory potential, I found that the 23 SSRs modulated promoter activity in six different cell lines, in a cell-type-dependent manner. 15 of 23 SSRs showed insulator activity in K562 cell line. Though many insulator proteins in *Drosophila* are known, the CCCTC-repeat binding factor, CTCF is the only insulator protein identified in vertebrates. Electrophoretic Mobility Shift Assays (EMSA) using nuclear extracts from human cell lines revealed that these SSRs bind specific proteins. Using affinity purification with proteomics, identifying novel SSR-binding proteins may provide insights into their roles as organisers and regulators of the genome.

P2095/B340

Set1/COMPASS Protects Active Euchromatin Against Heterochromatin Invasion by Disrupting Suv39/Clr4 Activity and Nucleosome Stability.

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Critical to the health and viability of the cell, is the ability to protect active euchromatin regions from invasion by gene-repressive heterochromatin in order to avoid deleterious transcriptional silencing. Heterochromatin, a conserved nuclear ultrastructure, is canonically found at constitutive loci including pericentromere and subtelomere regions where it has structural and genome defensive roles. In addition, facultative heterochromatin domains are also found interspersed in active euchromatin, where they regulate gene expression critical to creating and maintaining cell types. While it is known that heterochromatin and euchromatin harbor mutually exclusive information, the spatial encoding of and mechanisms by which euchromatic signals contain ectopic heterochromatin spreading remain opaque. Using a genetically encoded fluorescence-based spreading sensor system and global chromatin analysis, we investigated ectopic heterochromatin invasion in single fission yeast cells. We found that genes themselves can act as barriers to heterochromatin invasion and identified Set1/COMPASS as a gene-based, negative regulator of heterochromatin spreading via a genetic screen. Multi-locus validation confirmed that at least part of this genic barrier function is locally encoded by Set1 on actively transcribed genes and is not dependent on Set1 altering gene transcript levels. Genome wide, the major role of Set1 in protecting euchromatin from heterochromatin invasion occurs at small domains known as heterochromatin islands, which are interspersed in gene-rich euchromatin and regulate specifiers of cell fate. Set1 functions through at least two pathways to direct this gene protection activity - inhibiting catalysis by Suv39/Clr4 and disrupting nucleosome stability. Taken together, these results describe a mechanism for spatial encoding of euchromatic signals that regulate the expansion of heterochromatin domains.

P2096/B341

Heterochromatin Methylation Regulates Gnomic Stability.

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Heterochromatin methylation regulates genomic stability Cancer is typically considered a genetic disease where gene deletions or mutations are responsible for cancer-promoting changes within the cell. However, deregulation of epigenetic mechanisms is also prevalent in a variety of cancer contexts, and is increasingly appreciated as a contributing factor in tumorigenesis. Epigenetic modifiers regulate the placement and removal of covalent modifications on histones to orchestrate the recruitment of effector proteins and influence the compaction and accessibility of chromatin. In this way, deregulation of histone modifying enzymes can have dramatic consequences for gene expression and chromatin architecture. Epigenetic marks of constitutive heterochromatin are both increased and decreased in different subsets of cancers. However, while previous studies have demonstrated that *loss* of methylation that marks constitutive heterochromatin compromises mitotic fidelity, the impact of *increased* methyl marks at constitutive heterochromatin on cancer cell biology remains unclear. Here we show that alteration of both H3K9 and H4K20 methylation corrupts genomic instability. This loss of genomic stability resulting from altered heterochromatic methylation suggests that cancer-relevant epigenetic changes in constitutive heterochromatin regulation may underlie genomic instability seen in many different cancer contexts.

P2097/B342

Influence of the Epigenome on Chromatin Motions.

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Dynamic changes in chromatin organization maintain proper nuclear functions that include gene expression, DNA replication and genome maintenance. The movements of chromatin fibers themselves are thought to play important roles in the regulation of these fundamental processes. For instance, motions of DNA breaks are necessary for the onset of genomic translocations driving the initiation, progression and recurrence of certain cancers. Yet the mechanisms controlling chromatin mobility are still poorly understood. Here, we investigated the involvement of the epigenome in chromatin motions to better understand the functional significance of chromatin dynamics for genome function. We developed a method based on structured illumination to track and quantify the motions of chromatin microdomains in live cells. This method allow the tracking of photoactivated chromatin reporter (PACR) arrays produced with a diffractive optical element (DOE). This method allowed us to demonstrate a transient drop in chromatin motions after DNA damage induced by the radiomimetic drug bleomycin (BLM), or by ionizing radiations (IR). These results have been confirmed by computational simulations, showing that the motions observed are minimally influenced by noise. To further understand the role of the epigenome on chromatin motions, we investigated the motions of compacted chromatin in cells treated with histone demethylase inhibitors, and of relaxed chromatin in HDAC inhibitors-treated cells. Epigenetic sensors recognizing different histone modifications were used to assess the effect of drugs on chromatin statut, and to evaluate the motions of condensed compared to relaxed chromatin at the single cell level. Treatment of U2OS cells with dimethyloxalylglycine (DMOG), which inhibits H3K9 demethylases, led to an accumulation of H3K9me3 and showed a reduced chromatin diffusion. Hence, chromatin compaction is apparently leading to a measurable decrease in mobility. Our first results

suggest that the epigenome plays a role in the mobility of the chromatin. These results represent a first step toward the identification of chromatin motion regulators, and could help predict the biogenesis of genomic translocation.

P2098/B343

Modulation of Swi/Snf Mediated Developmental Programming by Bardet-Biedl Syndrome Proteins: Insights into the Etiology of Mckusick-Kaufman Syndrome and Congenital Heart Disease.

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Swi/Snf chromatin-remodeling complexes play crucial roles in development, stem cell biology and tumor suppression. Moreover, mutations in Swi/Snf chromatin remodeling proteins are associated in a significant way with congenital heart defects (CHD). Epigenetic regulators like Swi/Snf affect thousands of downstream target genes, making it difficult to identify the crucial target genes whose mis-regulation contributes to CHD. Smarcc1 (BAF155) and Smarcc2 (BAF170) subunits are core scaffolds that coordinate the assembly of all Swi/Snf complexes. Our published findings identified protein-protein interactions between Smarcc1 and the MKKS/BBS6 protein, a gene associated with both McKusick-Kaufman Syndrome, and Bardet-Biedl Syndrome. We show that BBS6 binds to and modulates the subcellular partitioning of SMARCC1 and that independent manipulation of either bbs6 or smarcc1 activity in zebrafish have shared and highly specific effects on the heart transcriptome, including effects on genes required for development of the heart. This study investigates the 1) biogenesis of the Swi/Snf complex and 2) the downstream developmental impacts relative to heart development. 1) Using transgenic zebrafish and knockout cell lines, we identified additional interactions between Smarcc1 and the other BBS-chaperonin proteins BBS10 and 12 as well as with the chaperonin CCT3, a subunit of the multi-protein CCT/TRiC complex, suggesting that the BBS chaperonin complex assists in the assembly of a SMARCC1-containing SWI/SNF subcomplex. 2) Congenital heart disease (CHD) affects ~2% of newborns each year and many, if not the majority of CHD defects, are the consequence of abnormal development of the outflow tract, OFT. We find that independent manipulation of either bbs6 or smarcc1 activity disrupts the formation of the OFT in zebrafish. We also find precocious activation of key genes involved in heart differentiation, including, *isl2b*, a gene required for outflow tract development. Swi/Snf complexes with different Smarcc1 to Smarcc2 ratios are associated with distinct cell states. Generally, ES-cells and some progenitors have Smarcc1-enriched Swi/Snf complexes; while more differentiated cells (post-mitotic and some progenitors) tend to be enriched for Smarcc2. Our work, showing precocious activation of developmental genes in the context of reduced Smarcc1 levels in zebrafish suggests that this dichotomy also applies to the developing heart.

P2099/B344

Chromatin Maturation Following Asymmetric Replication Coupled Nucleosome Assembly in *Drosophila*Adult Stem Cells.

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One of the major questions in developmental biology is how cells acquire different cell fates, and in particular how cell fate is determined during the asymmetric cell division of adult stem cells. Previously, our lab found that old and new histones H3 and H4 are segregated asymmetrically during the division of Germline Stem Cells. This could theoretically allow for direct reprogramming of the epigenome of the two cells. Mechanistically, this asymmetry is initially generated during DNA replication wherein old and

new histones are differentially deposited on sister chromatids due to both delayed lagging strand synthesis and coordinated replication fork progression. Following this dramatically asymmetric replication coupled nucleosome assembly, histone modifications as well as old and new histones are asymmetrically segregated on the forming sister chromatids. This generates, for example, a more than five-fold asymmetry in H3K27me3 segregating to the leading strand and H3K14ac towards the lagging strand as observed at the single molecule level on superresolved chromatin fibers. However, by mitosis the sister chromatids contain comparable levels of histone modifications. This predicts that there is a novel chromatin maturation program in stem cells, during which histone modification are selectively written on the histone modification depleted sister chromatid. The kinetics of this post-replicative maturation to globally symmetric levels are distinct for each histone modification as observed at both the single molecule level using chromatin fibers and globally in the whole nuclei using Edu pulse chases. While this program does generate comparable global levels for every observed histone modification, local asymmetries between sister chromatids can be observed at specific regions using superresolved mitotic chromosome spreads. Altogether, this suggests that stem cells not only have dramatic asymmetries in histone segregation, but that this is coupled to a unique chromatin maturation program that generates globally symmetric histone modification levels while specifically retaining certain asymmetries.

Chromatin and Chromosome Organization 2

P2100/B345

The Effect of Chromatin Structure on the Susceptibility to Double Strand Breaks and Choice of Repair Pathway Used.

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Cells are constantly being exposed to threats such as radiation, chemicals, and internal damage, the genome is at risk for DSB. If left unrepaired, the damage could result in mutations or cell death. When DSBs do occur, the cell has 3 pathways; apoptosis, non-homologous end joining, and homologous repair. The purpose of this study is to determine how DSB susceptibility and repair mechanism differs around the genome, and to determine if the chromatin environment affects these outcomes. In this experiment, tetracycline-inducible CRISPR will be used to induce DSBs in the gene regions of yeast cells to compare the DSB frequency and repair at different sites. DSB frequency was measured using quantitative PCR across break sites. DSBs would be reflected in a decrease in product as the amplification would increase at uncut sites and drop at cut sites. Preliminary data shows that repressed genes have higher levels of DSB at 3h of DSB induction than that of active genes. This suggests that chromatin environment of the cut site could affect the susceptibility of that site to DSBs. The high amount of DSB formation in the repressed genes could also be a result less efficient repair mechanisms than active genes. More trials are needed to investigate this relationship. Repair mechanism was measured using survival analysis, which tests if cells become resistant to the continuous DSBs at the cut site due to imprecise repair of the CRISPR recognition site. The percentage of survivor colonies (compared to total cells) estimates the mutation frequency of the gene. Preliminary results suggest that active chromatin locations might generally have fewer survivors, but more data and genomic loci must be examined. For example, a DSB in the coding region of the inactive *SEO1* gene (0.1 mRNA/cell), showed a high frequency of survivors after cutting. Survivor analysis had a mean percent survivors of 53%, with the lowest trial having 36% survivors and the highest trial having 92% survivors. Sequencing of the DSB region is underway to

explore this relationship as well as determine if there is a correlation between specific histone modifications and mutation frequency. This research will reflect how the different areas and structures of the genome are more susceptible to DSB and faulty repair which could lead to further mutation. Studying this deepens our understanding of genomic change in terms of how DSB repair can be altered by chromatin packaging.

P2101/B346

Misregulation of Linker Histone Variants in Glioblastoma.

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Glioblastoma (GBM) is the most common primary malignant brain tumor. The current standard of care includes maximal surgical resection followed by radiotherapy and chemotherapy. This disease remains deadly, with a dismal 5-year survival rate of 5.5%. In order for meaningful advances in the treatment, a thorough understanding of its phenotypic heterogeneity and associated epigenetic profile is required. Chromatin dysregulation is commonly observed in many cancers, including GBM. The role of linker histones, which play a key role in higher order chromatin organization, and which can dramatically alter the chromatin landscape, remains poorly understood. Previous reports have shown that loss of linker histone variant, H1.0, correlates with tumor progression and differentiation status. Interestingly, when we used patient samples to test H1 levels using a pan H1 antibody, a significant increase in H1 content was observed. Based on our TCGA database analysis of H1 variant expression in GBM, we found that H1 was significantly overexpressed in a variant specific manner. Since patient samples are limited, we used a model system of GBM cell lines to further investigate H1 variant overexpression induced chromatin changes in the GBM context. Following confirmation that the H1 variant protein content recapitulates the expression data, in patient samples and GBM cell lines, we used custom-made variant specific antibodies to, to generate the first comparative genome wide profile of H1 variant occupancy in GBM cell lines. Preliminary data shows an overall increase in specific binding, with a shift of H1 binding preference to specific genic regions. This change in binding preference was pronounced in the GBM cells especially at some gene regulatory regions like promoters, which may indicate a role of H1 in driving specific oncogenic changes. Taken together this indicates that variant specific changes in H1 expression and distribution may widely influence the chromatin landscape and also elicit GBM specific expression in the transcriptome.

P2102/B347

Molecular Mechanisms for the Protection of Sister Chromatid Cohesion at Mitotic Centromeres.

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Sister chromatid cohesion mediated by the ring-shaped multi-subunit cohesin complex is fundamental for precise chromosome segregation. Early in mitosis, while the bulk of cohesin is removed from chromosome arms by its antagonist Wapl, cohesin at centromeres is retained to ensure chromosome biorientation until anaphase onset. It remains to be determined how centromeric cohesin is protected against Wapl in mitosis. We find that the mitotic histone kinase Haspin associates with the cohesin complex through interaction with the cohesin regulatory subunit Pds5B, which is required to ensure proper centromeric cohesion (Zhou L et al., Curr Biol, 2017). Haspin also phosphorylates Wapl to inhibit the binding of Wapl to Pds5B, thereby antagonizing Wapl-mediated cohesin release (Liang C et al., EMBO Rep, 2018). Moreover, heterochromatin protein 1 (HP1) binds Haspin and facilitate its

centromeric localization, thereby linking centromere heterochromatin to sister chromatid cohesion (Yi Q et al., EMBO Rep, 2018). We reveal a positive feedback mechanism formed between the cohesin protectors Haspin and Sgo1 that ensures proper assembly of the functional inner centromere during mitosis (Liang C et al., J Biol Chem, 2019). We further dissect Aurora B kinase activity-dependent and -independent functions of the chromosomal passenger complex in regulating sister chromatid cohesion (Yi Q et al., J Biol Chem, 2019). Our studies provide mechanistic insights into how different factors in the complex centromere signaling network are specialized to meet the multiple challenges encountered on the road to accurate chromosome segregation in mitosis. We also suggest a causal link between centromeric cohesion defects and chromosomal instability in cancer cells.

P2103/B348

Live Cell Monitoring of Genome Variation Caused by Physical and Molecular Factors.

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Live cell monitoring of genome variation caused by physical and molecular factors Kuangzheng Zhu*[1], Yuntao Xia*[1], Jerome Irianto[1], Jason C. An drechak[1], Lawrence J. Dooling[1], Charlotte R. Pfeifer[1], and Dennis E. Discher[1] Molecular & Cell Biophysics Lab[1], Correspondence: *discher@seas.upenn.edu* * These authors contributed equally. Cancer cells and pluripotent stem cells frequently exhibit gains or losses of entire chromosomes and chromosome segments, with the typical terminal analysis by genomics suggesting that aneuploid variation is ongoing and especially variable within solid tumors. Here, we quantify aneuploidy-inducing perturbations by live cell fluorescence monitoring of changes in housekeeping gene expression from specific chromosomes in human cancer lines and normal diploid iPS cells. Inhibition of the spindle assembly checkpoint (SAC) and knockdown of DNA repair factors cause chromosome mis-segregation to increase several-fold above a low baseline level, and both perturbations also generate several-fold more rare fluorescent-null cells. Loss of chromosomes with loss of fluorescence is confirmed by single cell karyotyping, SNP arrays on stable isolated clones, and single cell transcriptomics that show downregulated expression of genes on lost chromosomes. Tumor growth in mice also generates rare fluorescent-null cells, as do teratomas generated from iPS cells, and flattened mitotic cells in tumors that are known to cause chromosome mis-segregation are mimicked in vitro by confinement and shown to produce fluorescent-null cells. Viability, selection, and altered expression can thus be tracked to reveal physical and molecular mechanisms of genome variation. Xia Y, Zhu K, *et al*. "Live cell monitoring for factors affecting genome variation." *bioRxiv* 50815 (2018).

P2104/B349

Daf2, a Novel RNA Binding Protein, Functions in Heterochromatin Assembly.

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Heterochromatin is a transcriptionally silenced domain in eukaryotic genome. Histone 3 lysine 9 methylation (H3K9me) is an epigenetic hallmark of heterochromatin. In fission yeast, RNA interference (RNAi) plays an important role in H3K9 methylation. Heterochromatin can be breifely transcribed during S phase. Heterochromatin transcripts are processed into siRNAs which promote H3K9 methylation in heterochromatin. Histone hypoacetylation mediated by histone deacetylases (HDACs) is another conserved epigenetic mark of heterochromatin. How RNAi and histone hypoacetylation are coordinated

during DNA replication to modulate heterochromatin assembly remains to be elucidated. Previously, we showed that Dpb4, a subunit of DNA polymerase epsilon complex, is important for the recruitment of HDAC to heterochromatin during S phase. Here using mass-spectrometry, we identified a novel RNA-binding protein Daf2 that interacts with Dpb4. We observed that H3K9me level at heterochromatin is compromised in the absence of Daf2. The siRNA generation is also defective in the *daf2* mutant. In addition, we found that Daf2 is required for the enrichment of Chp1, a subunit of RITS complex required for RNAi pathway, in heterochromatin. Importantly, we discovered that Daf2 physically interacts with heterochromatic transcripts as well as Chp1 in *vivo,* suggesting a direct role of Daf2 in RNAi-mediated heterochromatin formation. Furthermore, we showed that histone acetylation is increased whereas the association of the Sir2 histone deacetylase with heterochromatin is reduced in the deletion mutant of *daf2*. Togehther, our evidence suggests that Daf2 facilitates the assembly of heterochromatin by coordinating RNAi pathway and HDAC during DNA replication. This study discovers a novel role of DNA replication machinery in epigenetic inheritance of heterochromatin silencing.

P2105/B350

ALT Suppression Requires Localization of ATRX to PML Bodies Downstream of DAXX Binding of Histone H3.3.

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To counteract the end-replication problem, proliferating cells must enact a program of telomere maintenance. In normal dividing cells, telomeres are lengthened in an RNA-templated manner through the action of the ribonucleoprotein telomerase, but in 10-15% of tumors telomeres are lengthened through a DNA-templated process termed Alternative Lengthening of Telomeres or ALT. ALT is associated with mutations in the ATRX/DAXX/H3.3 histone chaperone complex, which is responsible for deposition of non-replicative histone variant H3.3 at heterochromatic regions of the genome including telomeres. Mutations in this complex result in aberrant telomeric chromatin, but it remains unclear whether H3.3 deposition specifically is required for ALT suppression. To address this question directly, we utilized the G292 cell line, in which ATRX is wild type but DAXX has undergone a fusion event with the non-canonical kinesin KIFC3[1]. The DAXX-KIFC3 fusion binds ATRX but fails to localize to nuclear PML bodies. Restoration of wild-type DAXX in G292 localizes ATRX and abrogates ALT. Using this model system, we tested the ability of a DAXX H3.3 binding mutant, DAXX-Y222E, to suppress ALT. The DAXX-Y222E mutant does not robustly suppress ALT-associated PML bodies (APBs), an ALT hallmark. Surprisingly, we found that DAXX-Y222E, though properly localized itself, fails to bind ATRX and localize it to nuclear PML bodies. To follow up, we tested a panel of DAXX variants with cancer associated missense mutations in the histone binding domain. We found that the DAXX mutants vary in their capacity to localize with ATRX, while a mutation that allows DAXX to bind H3.2 or H3.3 promiscuously forms foci with ATRX at frequencies similar to wild type DAXX. Notably, we find that the ability of the DAXX mutants to suppress APBs correlates with the extent to which they localize with ATRX. We speculate that these mutants represent a range of H3.3 binding affinities and propose that DAXX binding of H3.3 is upstream of complex formation with ATRX. These results imply a requirement of H3.3 for ALT suppression apart from its deposition and provide for the first time direct characterization of the biochemical defects of DAXX mutations found in human cancers. 1 Yost, K. E. et al. Rapid and reversible suppression of ALT by DAXX in osteosarcoma cells. Sci Rep 9, 4544, doi:10.1038/s41598-019-41058-8 (2019).

P2106/B351

Large-scale Chromatin Organization in Mammalian Cells.

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The cell nucleus is site of important processes such as DNA replication and repair and synthesis, maturation, and transport of RNA. These processes take place in non-membrane chromatin-containing dynamic domains. It is generally agreed that chromosomes occupy a particular region of the nucleus called chromosomal territories (CT). Conversely, the interchromatin compartment - the DNA-poor nuclear space that surrounds individual CTs - remains the subject of debate. The aim of our research is to analyze the distribution of DNA in the nuclear space, including large-scale three-dimensional (3D) chromatin arrangement in cell nuclei. We want to contribute to the discussion of chromatin architecture of the interphase nucleus. Light microscopy and fluorescent labels can provide an estimate of chromatin compaction and organization at specific genomic loci. For higher-resolution analysis, electron microscopy is required. We used a combination of focused ion-beam milling (FIB) and scanning electron microscopy (SEM) where a few-nanometers layer of material is removed from the top of the resin-embedded mammalian cells and the freshly cut block face is then scanned by electron microscope. This process is repeated automatically until a sufficient sample volume has been scanned. To visualize chromatin, the cells were treated by the pre-embedding method based on the extraction of RNA and blocking of carboxy and amino groups by methylation and acetylation where the chromatin, finally stained by uranyl, is observed as a highly contrasted structure. Our 3D reconstruction of chromatin arrangement showed occurrence of the condensed/inactive chromatin - decondensed/active chromatin - DNA-free interchromatin region organization of chromatin. Morphometric analysis of the nuclear regions containing DNA showed that DNA/chromatin occupies about half of the total nuclear volume, i.e. A significant part of nucleoplasm belongs to the interchromatin compartment. Furthermore, the analysis of distribution of DNA revealed approximately 40 % of chromatin domains associated with nuclear periphery (perinuclear chromatin) and about 30 % of chromatin domains associated with nucleolus (chromatin containing ribosomal genes). Our results demonstrate that the approach perpformed on nuclei with specifically contrasted chromatin offers an extraordinary opportunity for study of the nuclear architecture in *situ*. This work was supported by the Grant Agency of Czech Republic (19-21715S), by Charles University (Progres Q28). We acknowledge the Imaging Methods Core Facility at BIOCEV, institution supported by the Czech-BioImaging large RI projects (LM2015062 and CZ.02.1.01/0.0/0.0/16_013/0001775, funded by MEYS CR) for their support with obtaining imaging data presented in this work.

P2107/B352

CENP-A Preserves Genome Integrity during Alpha-satellite DNA Replication by R-loops Suppression at Human Centromeres.

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Centromeres are composed of repetitive alpha-satellite DNA with high sequence homogeneity between the higher order repeat (HOR) blocks. Homologous recombination has been postulated to play an active role in maintaining the repeat structure, but also implies that this process must be tightly regulated to avoid rearrangements, which could change the centromere size and strength, triggering genome instability (Black & Giunta, 2018). Using the centromere chromosome-orientation fluorescence in *situ* hybridization (Cen-CO-FISH; Giunta, 2018), we previously showed that the centromere-specific histone H3 variant CENP-A is important for maintaining centromere integrity (Giunta & Funabiki, 2017). Although these results indicate the presence of active mechanism that prevents rearrangements at alpha-satellites, the mechanism by which centromere instability is induced under these circumstances is unknown. Using an auxin-induced degron that allows rapid degradation of a tagged protein (Hoffmann et al., 2016), we found that removal of CENP-A from chromatin specifically upon entry in S-phase, but not during G2 or mitosis, leads to centromere instability accompanied with accumulation of γ-H2AX and increased recombination. Single molecule DNA replication assay shows impaired replication fork progression upon CENP-A depletion with accumulation of DNA-RNA hybrids at the centromere. HOR-associated alpha-satellites are actively transcribed into long non coding RNAs (McNulty et al., 2017). Indeed, replication-transcription conflicts may trigger centromere instability as depletion of CENP-A results in a significant increase in centromeric RNA during replication. Delayed centromere replication into mitosis resulted in frequent anaphase chromosome bridges, and ensuing chromosome breakage and translocations at the centromeric regions in the following cell cycle. In addition to serving as template for mitotic assembly of the kinetochore, our work points toward a novel role for CENP-A in coordinating replication and transcription of alpha-satellites to maintain the integrity of centromeres and chromosomes.

P2108/B353

Radiation Induced DNA Damage and Repair Effects on 3D Genome.

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The three-dimensional structure of chromosomes plays an important role in gene expression regulation and also influences the repair of radiation-induced DNA damage. Genomic aberrations that disrupt chromosome spatial domains can lead to diseases including cancer, but how the 3D genome structure responds to DNA damage is poorly understood. Here, we investigate the impact of DNA damage response and repair on 3D genome folding using Hi-C experiments on wild type cells and ataxia telangiectasia mutated (ATM) patient cells. Fibroblasts, lymphoblasts, and ATM-deficient fibroblasts were irradiated with 5 Gy X-rays and Hi-C was performed after 30 minutes, 24 hours, or 5 days after irradiation. 3D genome changes after irradiation were cell type-specific, with lymphoblastoid cells generally showing more contact changes than irradiated fibroblasts. However, all tested repair-proficient cell types exhibited an increased segregation of topologically associating domains (TADs). This TAD boundary strengthening after irradiation was not observed in ATM deficient fibroblasts and may

indicate the presence of a mechanism to protect 3D genome structure integrity during DNA damage repair.

P2109/B354

Confirmation of Genomic DNA Double-strand Break Hotspots in Forum Domains Termini.

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Forum domains are large fragments (typically 50-250 kb) of genomic DNA isolated from different species by pulsed field gel electrophoresis without any nuclease treatment (Tchurikov and Ponomarenko, 1992). Later was shown that such fragmentation non-random and represents some kind of genome functional and structural organization. Unique distribution pattern do not correlate to another chromatin organizations, like polytene banding, SARs/MARs, Pc domains. Significant part of forums contain group of genes with coordinate expression implying their functional role. Dedicated procedure to capture genomic DNA double strand breaks (DSBs) that contribute to the forum domains formation was developed (RAFT DNA library, Tchurikov *et al*., 2011). High throughput sequencing (NGS) of the library allowed precisely map and characterize DSB hotspots. Based on NGS data and more straightforward genomic DNA preparation we undertake study of particular hotspots to gather additional confirmation for DSBs in *D. melanogaster* S2 cells. The first approach implemented used real time PCR (qPCR) to quantify target containing a hotspot *vs* adjacent target free from DSBs. Cloning of a pair of targets in plasmid as a whole gives a DNA preparation that does not have any hotspot-specific DSBs and contains two targets in exactly one-to-one quantity. Hence, relative quantity of DSBs in genomic DNA can be inferred by discrepancy between PCRs of a pair of targets from expected at equimolar condition (no DSBs). It is obvious that a template DNA with DSB between a pair of PCR primers does not yield full length transcription products needed to sustain exponential growth. Transcripts growth and resources demand for such DNA templates expected to be linear and will not significantly distort exponential growth from true templates. QPCR data obtained with DNA intercalating dye from three hotspots, namely 2L:5, 3L:4.46, 3R:7.98, shows occurrence of DSBs at standard S2 growth conditions (z-score, $p < 0.05$). Interestingly, 2L:5 became more enriched with DSBs during physiological stress (heat shock, z-score, $p < 0.05$). In the second approach primer extension procedure on genomic DNA was employed. Radiolabeled primer elongating on genomic DNA gives single stranded products to be abrupted at 5` end of a DNA break. Preliminary results are somewhat different from RAFT library sequencing results. Typically, NGS of the library gives significant break counts on full hotspot width (some hundreds bps). However, with primer extension on 3R:7.98 and 2L:5 minus strands much narrower genomic DNA hotspots was observed. Details to be discussed during the meeting. The reported study was funded by RFBR according to the research project No. 18-34-00892.

P2110/B355

The Disordered Regions of Heterochromatin Protein-1 Paralogs Regulate Dna Compaction and Dna Induced Phase Separation.

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Chromatin structure is related to transcriptional output. Repressed genes are sequestered from active genes into regions of dense compacted DNA. These dense domains in *vivo* are associated with Heterochromatin protein-1 (HP1). HP1 concentrates DNA and chromatin into phase separated droplets in *vitro*, suggesting a potential mechanism of DNA organization in *vivo.* We utilize DNA curtains and

confocal microscopy to investigate HP1-mediated compaction of DNA and reconstituted chromatin substrates. We identify the key regions of the HP1α paralog required for multivalent interactions and phase separation. The internal hinge region of HP1 was purified and found to compact DNA faster and induce DNA-driven droplet formation at lower concentrations than the full length protein, suggesting autoregulation. We find this regulation is provided by the disordered C-terminus of HP1, as truncation of the C-terminal extension leads to increased compaction rates and lower critical concentrations of DNA-driven droplet formation. Further, the N-terminal extension exhibits positive regulation. Finally, we find that sequence differences in the disordered regions of the three human paralogs (α, β, γ) regulate DNA compaction, suggesting the differential association with transcription states in *vivo* could be due to intrinsic biochemical differences.

Regulatory and Non-Coding RNAs

P2111/B357

Novel Scarna Processing and the Formation of Regrnps.

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Non-coding RNAs are important in cellular function. A specific class of non-coding RNA, small Cajal Body-specific RNA (scaRNA), is responsible for guiding modification of small nuclear RNA (snRNA) in the Cajal Body (CB). CBs are subnuclear domains that take part in the biogenesis of many different types of ribonucleoproteins (RNPs), including scaRNPs. Modification of snRNA by scaRNPs is necessary for proper snRNP activity and spliceosome function. Since 2004 only 3 scaRNAs (scaRNA 2, 9, and 17) out of the 30 known scaRNAs have been found to be processed into fragments that accumulate in the nucleolus. We hypothesize that these RNA fragments complex with proteins and form regulatory RNPs (regRNPs). We further hypothesize that the scaRNA fragment component of regRNPs base pairs with the small nucleolar RNA (snoRNA) component of small nucleolar RNPs (snoRNPs). Interaction of regRNPs with snoRNPs alters the rRNA modification activity of snoRNPs. Differences in the level of rRNA modification in ribosomes is the main contributor of ribosome heterogeneity. It is possible that rRNA modification can be altered in different disease states resulting in ribosomes with differential fidelity and regRNPs are important components in this process. We have found by Northern blotting that an additional 5 scaRNAs (scaRNA 5, 6, 7, 10, and 12) can be processed into smaller fragments. Unlike the fragments generated from scaRNA 2, 9, and 17, the fragments from scaRNA 5, 6, 7, 10, and 12 are all around 200 nts in length. We speculate that these fragments were not detected previously due to the positioning of the probes used to detect the scaRNAs. Interestingly, all 8 processed scaRNAs contain box C/D motifs and are greater than 200 nts in length compared to scaRNAs that we have found to not be processed such as scaRNA 13 (tandem H/ACA) and scaRNA 28 (194 nts). The exact mechanism for the internal processing of scaRNAs is unknown and is a current topic of investigation. Like the fragments derived from scaRNA 2, 9, and 17, we hypothesize that scaRNA 5, 6, 7, 10, and 12 processed fragments form regRNPs that can impact scaRNP modification of snRNA or affect the level of rRNA modification in the nucleolus through interactions with the snoRNA component of snoRNPs. Further work will include examining the impact of regRNPs on snoRNP and scaRNP activity and defining the components that control scaRNA processing.

P2112/B358

Novel Role of Trna-derived Fragments in Protein Arginylation.

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Posttranslational modifications are fundamental processes that expand the functional diversity of the proteome. Arginylation mediated by arginyltransferase ATE1 is a posttranslational modification of emerging importance that regulates mammalian embryogenesis, cell migration, and normal brain function, and has been recently proposed as a global biological regulator. However, the molecular mechanism of arginylation is elusive. Our preliminary results showed that while arginylation is very specific to Arg-tRNA^{Arg} its reactivity is similar towards all mouse tRNA^{Arg} isoacceptors, suggesting ATE1 interaction with tRNA^{Arg} is determined by its targeting toward the tRNA^{Arg} acceptor stem. In support, tRNA^{Arg} stem structures conjugated to Arg are capable of mediating in *vitro* arginylation. Since these stem structures bear resemblance to predicted tRNA-derived fragments (tRF) known to be generated in cells via the action of RNaseT2, we next tested the possible involvement of tRF^{Arg} in arginylation. Our data show that RNaseT2 is capable of cleaving the arginyl-tRNA^{Arg}, and this cleavage product is capable of mediating arginylation with similar efficiency to the intact Arg-tRNA^{Arg}. Moreover, our tRNA- and tRF-Seq results showed that the ratio of tRNA^{Arg} to tRNA^{Arg}-derived tRF is significantly higher in Ate1 knockout cells compared to wild type, suggesting that lack of arginylation inhibits tRF^{Arg} generation. We propose that tRF^{Arg} can potentially be dedicated solely to arginylation, which enables this modification to maintain its levels even in the high background from protein synthesis. Our study suggests that generation of physiologically important tRFs can potentially play a critical role in a switching mechanism between protein translation and arginylation in vivo, and points to the new role of tRFs in protein modifications.

P2113/B359

A Complex Containing Mettl5 and Trmt112 Installs*N*^{6}-methyladenosine on 18s Ribosomal Rna.

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Ribosomal RNAs (rRNAs) have long been known to carry modifications, including numerous sites of 2'*O*-methylation and pseudouridylation, as well as *N*^{6}-methyladenosine (m^{6}A), *N*$^{6,6-}$dimethyladenosine, and others. While the functions of many of these modifications are unclear, some are highly conserved and occur in regions of the ribosome critical for mRNA decoding. Both 28S rRNA and 18S rRNA carry m^{6}A, and while ZCCHC4 has been identified as the methyltransferase responsible for the 28S rRNA m^{6}A site, the methyltransferase responsible for the 18S rRNA m^{6}A site has remained a mystery. We have identified METTL5 as the enzyme responsible for installing m^{6}A at position 1832 of human 18S rRNA. The small multifunctional methyltransferase subunit TRMT112 enhances METTL5 stability and activity, as demonstrated both by protein expression and in vitro activity assays. This m^{6}A site is located on the 3' end of 18S rRNA, which may become surface exposed in the context of a translating ribosome and thus may play a regulatory role in translation. In addition to its role as an rRNA methyltransferase, sequencing of METTL5-bound RNAs suggests that it may have other RNA substrates. While recent work has focused heavily on m^{6}A modifications in mRNA and its roles in mRNA processing and translation, deorphanizing putative methyltransferase enzymes is revealing previously unappreciated regulatory roles for m^{6}A in noncoding RNAs as well.

P2114/B360

A Novel Role for the Rab-6.2 GTPase in Regulating Small RNAs and Ras Signaling in *C. Elegans*.

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Epidermal growth factor receptor (EGFR)/Ras/MAPK signaling promotes cell growth and can drive tumorigenesis when overactive. *Caenorhabditis elegans* vulva development can model this pathway as signaling from the EGFR homolog LET-23 induces the vulval cell fate in vulva precursor cells (VPCs). The RAB-6.2 GTPase has an established role in retrograde trafficking, and we initially hypothesized that it would play a similar role in the VPCs to regulate EGFR trafficking and Ras signaling. While EGFR trafficking is unaffected in *rab-6.2(-)* mutants, we found that a *rab-6.2* deletion suppresses the under-induced phenotypes of signaling-defective mutants, suggesting an antagonistic function of RAB-6.2 in Ras signaling. In addition, *rab-6.2(-)* mutants display decreased expression of transgenic arrays, hinting at a role for RAB-6.2 in transgene silencing, a process regulated by small RNAs. In fact, VPS-52, a RAB-6.2 effector, promotes activity of the *let-7* microRNA family, known to repress Ras in *C. elegans* and mammals. We hypothesize that RAB-6.2 supports the activity of the *let-7* miRNA family, and through this negatively regulates Ras signaling. In line with this, we found that deletion of the *let-7* family member *mir-48* suppresses the under-induced phenotype of signaling-defective mutants, suggesting that *mir-48* antagonizes Ras signaling, in line with a RAB-6.2/*let-7* family regulatory axis. Furthermore, *rab-6.2(-)* enhances the usually weak phenotypes of *daf-19*, *cel-1*, and *unc-73* RNAi, suggesting hypersensitivity to RNAi, which can result from increased availability of small RNA machinery in a background with reduced miRNA activity. Overall, our results pose RAB-6.2 as a regulator of small RNAs and Ras-mediated vulva induction, and suggest that miRNAs underlie this. These findings could expand our understanding of the functions of RAB GTPases and provide insight into the spatial regulation of small RNA biogenesis and function.

P2115/B361

Defining the Molecular Mechanism of a Long Noncoding RNA That Enhances Intestinal Epithelial Antiparasitic Defense.

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The intestinal parasite *Cryptosporidium parvum* is a major cause of diarrheal disease, particularly in developing countries, and additionally remains a significant AIDS-related opportunistic pathogen. Currently, no fully effective treatment exists. Long noncoding RNAs (lncRNAs) can act as key modulators of diverse cellular processes through interactions with DNA, RNA, and proteins. LncRNAs have been identified that play a role in the innate inflammatory response, however, the functional mechanism has been elucidated only in a select few. The objective of this study was to identify lncRNAs induced upon *C. parvum* infection, determine whether these lncRNAs contribute to epithelial defense, and if so, elucidate the underlying molecular mechanism. To this end, a microarray was performed in the intestinal epithelial cell line, IEC4.1, infected with *C. parvum to* compare with uninfected controls. An alysis of the microarray identified 1,385 significantly differentially expressed lncRNA upon *C. parvum* infection. One lncRNA, referred to as lncRNA-25B here, was induced 2.3 fold upon *C. parvum* infection in *vitro*, 2.8 fold using a well-established cryptosporidiosis infection model in neonatal mice, and 2.1 fold using an *ex vivo* murine enteroid model. Interestingly, overexpression of lncRNA-25B significantly

reduced *C. parvum* infection burden compared to empty vector control, while knockdown of lncRNA-25B significantly increased the infection burden compared to a scrambled siRNA. RNAseq analysis revealed knockdown of lncRNA-25B resulted in the dysregulation of defense genes, many of which were target genes of NF-κB, suggesting a role for lncRNA-25B in the regulation of NF-κB signaling. RNA immunoprecipitation demonstrated a physical interaction between NF-κB p65 and lncRNA-25B, and that this interaction is increased 2.7 fold following *C. parvum* infection. In conclusion, these experiments have identified a *C. parvum* induced lncRNA which was found to impact the parasite infection burden through interactions with NF-κB p65 and subsequent modulation of defense genes. Future experiments will investigate how lncRNA-25B may facilitate NF-κB signaling to promote epithelial antimicrobial defense.

P2116/B362

Localized Regulation of RNAi-lncRNA Interactions by Epithelial Adherens Junctions.

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The adherens junctions (AJs) are essential architectural elements of epithelial tissues. Compromised junctional integrity is a common precursor to colon cancer. Recently, we identified a novel mechanism whereby the AJs of non-transformed colon cells recruit the microprocessor and RISC, core elements of the RNAi machinery, as well as miRNAs, to suppress oncogenic mRNAs. Knockdown of the AJ component, PLEKHA7, disrupts this RNAi-mediated signaling program, leading to pro-tumorigenic cell transformation. Interestingly, PLEKHA7 RNA-CLIP and subsequent RNA-Seq analysis identified association with numerous long non-coding RNAs (lncRNAs). While a number of lncRNAs have been associated with tumorigenesis, the underlying mechanisms of their regulation during tumor progression are still unclear. As lncRNAs can interact with the RNAi machinery, we hypothesize that the AJs regulate lncRNAs via this localized RNAi mechanism. Examination of PLEKHA7-depleted cells by RNA-seq revealed differential expression of 49 junction-associated lncRNAs. From this set, the top upregulated lncRNA is MIR17HG, an oncogenic polycistronic host transcript of the miR-17-92 cluster. Junctional localization of MIR17HG was confirmed by RNA-FISH. Adherens junctions destabilization by E-cadherin knockdown also led to the upregulation of MIR17HG, demonstrating the role of junctional integrity in MIR17HG suppression. Data from AGO2 knockdown, antago-miR, and miRNA mimetic experiments show that PLEKHA7 suppresses the levels of the MIR17HG transcript through the junction-associated RISC and miRNAs. PLEKHA7 depletion also results in increased levels of a subset of MIR17HG hosted miRNAs, including miR-19a and miR-19b, both known cancer promoters. We observe extensive mis-localization or loss of PLEKHA7, co-existent with mis-localization of junctional RNAi machinery, in colon cancer tissues and cell lines. Ectopic expression of PLEKHA7 in aggressive colon cancer cells that lack endogenous PLEKHA7 expression, suppressed MIR17HG levels, as well as anchorage independent growth. We are currently examining the role of MIR17HG as a functional intermediate of PLEKHA7's loss on colon cell transformation. In summary, our data point towards a novel mechanism of lncRNA regulation that tethers epithelial architecture with cell behavior.

P2117/B363

Non-coding Rna as Modulator of *V*irulenceIn Streptococcus Pneumoniae and Potential Role as Epidemiology Biomarker.

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Small noncoding RNAs (sRNAs) play important roles in gene regulation in both prokaryotes and eukaryotes. As the dynamics of functioning of CiaRH (the two-component regulatory system which is responsible for β-lactam resistance, maintenance of cell integrity, competence and virulence) of Streptococcus pneumoniae is poorly studied, the aim of our study was to define role of potential of smallRNA in CiaRH mechanism and overall in pathogenesis of pneumococcal infection. Matherials and methods: we isolated RNA from Streptococcus pneumonia TIGR4 strain, then transcriptional start points were determined by 5'-RACE as described previously using the same RNA adapter and gene-specific primers, in vitro CiaR binding, and in vivo analysis of CiaR-mediated regulation. Results: promoters were identified to be directly controlled by the response regulator CiaR. The genes that are transcribed from these promoters included ciaRH, loci that are predicted to be involved in the modification of teichoic acids (lic), in sugar metabolism (mal, man), stress response (htrA), chromosome segregation (parB), protease maturation (ppmA) and unknown functions. Ten of the regulon are activated by CiaR, and one was found to be controlled negatively. Five strongest promoters of the CiaR regulon drive expression of small RNAs. These small RNAs, designated csRNAs for cia-dependent small RNAs, are non-coding, between 60 and 71 nt in size, and show a high degree of similarity to each other. Conclusion: many of the identified sRNAs have important global and niche-specific roles in virulence what constitute the most comprehensive analysis of pneumococcal sRNAs and provide the first evidence of the extensive roles of sRNAs in pneumococcal pathogenesis.

P2118/B364

Regulatory Role of Xist Rna during Mouse Hematopoiesis.

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Xist noncoding RNA is essential for initiation of X chromosome inactivation (XCI) which results in transcriptional silencing of one of the two X chromosomes in female cells equalizing X-linked gene expression between female and male mammals. Initiation of XCI relies on several epigenetic processes including recruitment of Polycomb Repressive Complex 2 and subsequent deposition of repressive histone modification, Histone 3 Lysine 27 trimethylation (H3K27me3). XCI is stably maintained with continuous Xist expression in epiblast cells and in adult tissues. Nevertheless, we have little understanding on the significance of Xist expression during the XCI maintenance and how this process influences lineage specification or cellular states during development. In mice, hematopoietic lineage develops around embryonic day 10.5 representing an ideal developmental stage to study affects of Xist during XCI maintenance. Notably, targeted deletion of Xist in hematopoietic stem cells (HSCs) results in female-specific hematopoietic defects including expansion of LSK+ stem cells, depletion of long-term HSCs and maturation and proliferation defects in mature cells. Xist mutant mice exhibit bone marrow deficiency coupled with 100% penetrant blood cancers in the form of mixed myeloproliferative and myelodysplastic syndrome (MPN/MDS). Here, we investigated the cellular basis for hematopoietic

defects that are observed in blood-specific Xist knockout mice and examined how Xist deficiency influenced the transcriptome and distribution of H3K27me3 during hematopoiesis. We found that Xist deletion leads to distorted proportion of LSK+ stem cell subpopulations, which presented altered cell cycle activity. Notably, Xist mutant LSK+ stem cells showed significant upregulation of X-linked genes and depletion of H3K27me3 on the Xi suggesting that Xist is necessary for maintenance of transcriptional silencing and PRC2 activity on the Xi. These results suggested that Xist-mediated maintenance of XCI is specifically critical for hemostasis and cell cycle regulation of LSK+ stem cell population. We propose that in LSK+ stem cells, Xist deficiency leads to differential expression of X-linked genes that trigger downstream signaling events resulting in altered cell cycle activity and subsequent bone marrow deficiency. Future studies addressing the functional relevance of individual X-linked genes that are differentially regulated in Xist mutant LSK+ stem cells will provide mechanistic insights causally linking XCI to female-specific blood malignancies.

P2119/B365

Dicier: a Fine-tuned Bioinformatics Pipeline to Detect Micro-RNA Chimeric Reads.

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MicroRNAs are short non-coding RNAs (~22nt) that associate with Argonaute proteins and mediate regulatory silencing of target RNAs. The identification of RNA targets is guided by complex sequence and structured interactions between miRNA loaded AGO protein and the target RNA. Binding of identified targets can lead to repression of translation or even deadenylation and RNA degradation. Each miRNA has potentially thousands of binding sites on the transcriptome, and each mRNA contains dozens of potential miRNA binding sites. The Ago-miRNA regulatory function plays central roles in several processes including tissue development and various diseases such as diabetes, cancer and heart disease among others. Cross-Linking Immuno-Precipitation (CLIP) is a molecular biology method that has been extensively used in recent years to locate transcriptomic regions where proteins bind mRNA. Next-Generation Sequencing (NGS) combined with CLIP allowed a massive identification of those binding sites (CLIP-Seq). A major drawback of CLIP-Seq techniques when dealing with AGO-miRNA binding is that it is impossible to know which miRNA sequence, out of hundreds expressed, is responsible for each binding. Recently, an improvement of CLIP-Seq techniques was achieved with crosslinking, ligation, and sequencing of hybrids assay, which increased the yield of hybrid sequences between mRNA and its miRNA binding sites (chimeric sequences). These sequences contain both parts of the miRNA and target site thus unambiguously defining the miRNA responsible for each hybrid binding. However, the identification of chimeric sequences from CLIP experiments lacks up-to-date bioinformatics strategies. We have developed ‘DICIER’, a pipeline for the identification of miRNA:binding site chimeric reads from CLIP-Seq experiments. ‘DICIER’ maps raw reads to the reference genome and retrieves ambiguous alignments as candidate chimeric sequences. Those chimeric sequences are broken into two subsequences to first match a database of miRNA sequences and then to find the corresponding miRNA-binding site at the genomic level. ‘DICIER’ output a table of miRNA and mRNA binding-site connections, which can be used as a reference for further studies. We benchmarked ‘DICIER’ on both CLASH and CLIP-Seq public datasets, and we identified N=250000 candidate miRNA-mRNA binding-sites connections. Despite the majority of those connections were originated from CLASH experiments, the increased sensitivity of ‘DICIER’ allowed the identification of chimeric reads from CLIP-seq in a proportion of up to 5% of total sample reads. **This research has been financially supported by the Ministry of Education, Youth and Sports of the Czech Republic under the project CEITEC 2020 (LQ1601).**

P2120/B366

The Role of LncRNA-75 in TGF-β Signaling and Breast Cancer Progression.

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The progression of breast cancer to the metastatic stage has severe clinical implications, adversely affecting survival compared to non-metastatic growths. Previous research has implicated long noncoding RNAs (lncRNAs)—defined as noncoding RNA transcripts greater than 200 nucleotides in length—in cancer progression, although the majority of lncRNAs have not been well characterized. We performed microarray analysis to identify lncRNAs exhibiting significant expression changes in cancerous cells, and which functioned in the context of the TGFβ signaling pathway. This pathway was selected because it plays critical, yet disparate roles in cancer progression by suppressing cell proliferation while simultaneously promoting the epithelial-mesenchymal transition (EMT), a hallmark of metastasis. From this screen, lncRNA75 was selected for further study as it was significantly upregulated. To better understand the role of lncRNA75, we performed a siRNA knockdown in *vitro in* MCF10A cells, and observed cell cycle arrest in lncRNA75 siRNA KD cells. This cell cycle arrest was increased upon addition of TGFβ ligand, suggesting an additive effect. To determine if lncRNA75 is directly induced by TGFβ, we cloned the lncRNA75 promoter into a luciferase reporter plasmid and measured luciferase activity following treatment with SB431542 (a TGFβ inhibitor) and TGFβ. We observed a significant increase in luciferase activity following TGFβ treatment, suggesting that the promoter contains one or more TGFβ binding elements, and isolated this binding region to a 2kb segment of the promoter. This also suggests that lncRNA75 is upregulated in response to TGFβ signaling, as shown by the dramatic increase in promoter activity. To determine a mechanism by which lncRNA75 might affect cancer progression, we performed RNA immunoprecipitation and determined that YBX1, an RNA binding protein with oncogenic properties, interacts with lncRNA75. In summary, we have identified a novel lncRNA, lncRNA75, that suppresses cell proliferation when depleted and is upregulated by TGFβ through interaction with the lncRNA75 promoter. We have also identified an interaction with the oncogenic protein YBX1. Future work, including in *vivo* experimentation and analysis of epithelial-mesenchymal transition markers, will allow us to further clarify the role of lncRNA75 in cancer progression and develop it as a potential therapeutic target.

P2121/B367

Deciphering Circular Rna Axes in Early-onset Breast Cancer Using a Breast Epithelial Risk-progression 3d Culture Model.

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One of known circular RNAs (circRNAs) functions is to sponge microRNAs (miRNA). Studies reported dysregulated mRNA-circRNAs-miRNAs axes as biomarker signatures in breast cancer (BC). However, signatures in early-onset BC have not been characterized, while its incidence increased drastically worldwide. We used our 3D breast epithelial risk-progression HMT-3522 S1 (S1) culture model to

identify dysregulated post-transcriptional axes. S1 (non-neoplastic) cells form fully-polarized epithelium and closely mimic normal*in vivo* mammary epithelial morphology, while (pre-tumorigenic) Cx43KO-S1 (S1 silenced with shRNA for gap junction Cx43) lose epithelial polarity, multi-layer and mimic tumor-initiated in *vivo* mammary epithelial morphology. We performed circRNA microarray (Arraystar Human circRNA Array V2) and detected 75 significantly upregulated and 46 significantly downregulated circRNAs (Fold Change > 2) in 6 samples of Cx43KO-S1 versus S1 cells in 3D. We then selected 10 upregulated and 8 downregulated circRNAs for validation. Of these, hsa_circ_0007961 and hsa_circ_0081481 were most significantly upregulated while hsa_circ_0060055, hsa_circ_0005185, hsa_circ_0083442 and hsa_circ_0077755 were most significantly downregulated by RT-qPCR. We focused on "possible" sponging activity of validated circRNAs to their predicted (*in silico*) target miRNAs, and compared their dysregulation to our validated published miRNA datasets (Nassar et al., 2017). When circRNAs are upregulated, they downregulate function of their sponged miRNAs, hence preventing them from deregulating their target mRNAs. For upregulated **hsa_circ_0007961** (originating from **SPRED2** mRNA), its predicted **miR-99a-3p** was indeed downregulated in young Lebanese BC patient tissues and matched US patients, and predicted miR-653 possessed treatment potential in BC cells. For the second upregulated **hsa_circ_0081481** (originating from **FBXO24**mRNA), its predicted **miR-3960** was exclusively dysregulated in young Lebanese BC patients. For downregulated hsa_circ_0077755 (originating from **Cx43** mRNA), its predicted **miR-182** was commonly upregulated in young Lebanese and matched US patients and predicted **miR-203a-3p** was reported as promising prognostic biomarker in triple negative BC. Additionally, all predicted miRNAs targeted by all detected circRNAs were mostly enriched in pathways in cancer. Thus, our 3D risk-progression culture model might help delineate post-transcriptional axes to better understand early-onset BC initiation. We reveal three validated axes: i) SPRED2/hsa_circ_0007961/miR-99a-3p or miR-653-5p, ii) FBXO24/hsa_circ_0081481/miR-3960 and iii) Cx43/hsa_circ_0077755/miR-182 or miR-203, which could serve as potential biomarker signatures in early-onset BC.

P2122/B368

Determining Long Noncoding RNA Interactions in Stem Cells Using Automated Image an alysis and Single Molecule RNA-fluorescent in Situ Hybridization.

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Human diseases are often caused by abnormal biological processes within specific cell types or individual cells within a tissue, yet most studies have focused on cell populations and ignored possible differences between individual cells. One reason is a lack of easily implemented and broadly applicable experimental and computational approaches for microscopy image acquisition and analysis. We propose an approach in which we optimize the experimental data acquisition prior to analysis to generate high quality images that are easier to analyze computationally. The images are then processed and analyzed in parallel to determine cellular and nuclear boundaries, which allows for single-cell quantification of features of interest. We applied our approach to investigate mammalian antisense long noncoding RNAs (lncRNAs) Tsix and Xist. These transcripts have been previously shown to have inhibitory interactions, though multiple possible mechanisms have been proposed. By utilizing two-color, single-molecule RNA-FISH and our image acquisition and analysis pipeline, we were able to quantify Tsix and Xist at single-cell and single-molecule resolution in more than 8000 cells. Our analysis revealed that there was no significant colocalization of Xist and Tsix molecules, which provides evidence against previously proposed mechanisms of inhibition involving the binding of the two transcripts. We also found that

anticorrelation between Xist and Tsix was only apparent when investigating the sites of transcription, which suggests mechanisms of inhibition in *cis* based on the act of transcription. This data demonstrates the importance of single-cell, allele-specific analysis when investigating noncoding RNA interactions. Our generalizable pipeline can be applied for further quantitative investigation of antisense transcription for other genes across diverse species, cell types, and imaging modalities.

P2123/B369

Identification of Serum MicroRNA Signature Associated to Interstitial Lung Abnormalities in Asymptomatic Subjects > 60 Years Old.

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Background: Interstitial lung abnormalities (ILAs), diagnosed by high resolution computed tomography, are observed in around 9% of older asymptomatic subjects, mainly smokers. Some of them may progress to lung fibrosis. The mechanisms associated to ILAs development and progression are unknown. Identifying biomarkers of this subclinical disease status is relevant for early diagnosis and to predict outcome. Circulating microRNAs (miRNAs) have emerged as regulators of pathogenic mechanisms and potential non-invasive biomarkers for lung diseases. Levels of circulating miRNAs in ILA are still unknown. *Aim*: to identify the serum miRNAs expression pattern associated to ILA in a cohort of respiratory asymptomatic Mexican subjects older than 60 years. *Methods*: from a lung-aging study cohort in Mexico City (n=826), we identified patterns of ILA in 81 subjects that were compared with 112 asymptomatic individuals randomly selected from the same cohort (Ctrl). For the screening phase, serum miRNA profiles were analyzed in a cohort of 24 subjects with ILA and 24 controls, by using TaqMan MicroRNA Arrays containing probes for 366 miRNAs. Differentially expressed miRNAs in ILAs vs. Ctrl were identified ($p \leq 0.05$ and FoldChange > 2) and selected candidates were validated with RT-qPCR in the serum of 57 ILAs and 88 Ctrl (independent cohort). *Results*: We found seven serum miRNAs significantly up-regulated in ILAs compared to Ctrl: miR-502-3p ($p < 0.0001$), miR-193a-5p ($p < 0.0001$), miR-200c-3p ($p < 0.003$), miR-16-5p ($p < 0.003$), miR-34a-5p ($p < 0.005$), miR-126-3p ($p < 0.004$) and miR-21-5p ($p < 0.002$). Common pathways regulated by these miRNAs include TGF-beta, Wnt, mTOR, Insulin, MAPK signaling and cancer pathways. In addition, several studies have associated overexpression of miR-200c, miR-34a-3p and miR-21 to senescence. Receiver-operator characteristic (ROC) curve analysis indicated that miR-193a-5p (AUC: 0.750), miR-502-3p (AUC 0.710) and miR 21-5p (AUC 0.855) have acceptable diagnostic value. *Conclusions*: This study provides potential miRNAs candidates as non-invasive biomarkers and molecular targets to better understand the putative pathological mechanisms associated to ILA.

Nuclear Envelope 2

P2124/B371

VAPB Proteomic Mapping by Rapamycin-dependent Targeting of APEX2 Identifies Binding Partners at the Inner Nuclear Membrane.

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VAPB (vesicle-associated membrane protein-associated protein B) is an integral endoplasmic reticulum (ER) protein that is present at several contact sites of the ER. We now show by immunoelectron microscopy and a rapamycin-based dimerization assay that VAPB also localizes to the inner nuclear membrane (INM). The engineered ascorbate peroxidase (APEX2) has been effectively employed in mammalian cells to identify protein-protein interactions.Using a modified APEX2-approach with rapamycin-dependent targeting of the peroxidase to a protein of interest, we identified proteins that are in close proximity to VAPB. In combination with stable isotope labeling with amino acids in cell culture (SILAC), followed by co-immunoprecipitation assays, we confirmed many well-known interaction partners of VAPB at the ER and also identify proximity partners at the INM. Hence, rapamycin-APEX2-mediated proximity labeling of VAPB neighboring proteins provide insights into the VAPB interactome at the ER and the INM.

P2125/B372

Barrier to Autointegration Factor: a Well-regulated Mechanism to Repair Nuclear Ruptures.

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The nuclear envelope (NE) enables a well-regulated and critical barrier between the cytosol and nucleus. Transient loss of this cytoplasmic/nucleoplasmic barrier via mechanical forces exerted on the NE and/or intrinsic loss of structural integrity compromises genomic integrity. Remarkably, cells are capable of rapidly repairing ruptures of the NE. Barrier-to-autointegration factor (BAF), a DNA-binding protein involved in post-mitotic NE reformation and cytosolic viral regulation, rapidly and substantially binds to nuclear DNA exposed to the cytosol upon rupture. This accumulation of BAF subsequently recruits components involved in NE reconstitution, including LEM-domain proteins, membranes, and the ESCRT-III membrane repair complex. Loss of BAF prevents recruitment of these components, resulting in a failure to restore the barrier function of the NE. Similarly, simultaneous loss of multiple transmembrane

LEM-domain proteins, but not the ESCRT-III complex, also prevent repair of NE ruptures. BAF's DNA-binding is regulated via phosphorylation by vaccinia-related kinases (VRKs). Loss of VRK1, but not VRK2, leads to prolonged hyperaccumulation of BAF at nuclear ruptures, impacting the repair process and suggesting that BAF may also function to compact DNA at rupture sites to mitigate the loss of subcellular compartmentalization, effectively acting as a 'clotting factor'. Collectively, these studies reveal a well-regulated response to nuclear ruptures that enables cell viability following this seemingly catastrophic cellular injury.

P2126/B373

Nuclear Rupture as a Potential Shared Mechanism of Nuclear Envelope-associated Progeria.

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Specific mutations in any one of the genes encoding A-type lamins, barrier-to-autointegration factor (BAF), and LEMD2 have been reported to cause progeria, a premature aging disease with variable phenotypes. These three nuclear envelope proteins are all capable of physically interacting with each other. It is therefore reasonable to hypothesize that there is a shared mechanism of disease underlying these nuclear envelope-associated progerias. We have recently demonstrated that BAF localizes to sites where the nuclear envelope is ruptured and is required to recruit A-type lamins and transmembrane LEM-domain proteins, including LEMD2, to repair the nuclear envelope. Nuclear ruptures result from exposure to mechanical forces on the nucleus and/or a weakened nucleoskeleton that compromises the integrity of the nuclear envelope and leads to the loss of nucleocytoplasmic compartmentalization. Under normal conditions, nuclear ruptures are rapidly repaired to stop the unrestricted exchange of cellular constituents between the cytosol and nucleus that is reported to, among other likely consequences, lead to DNA damage and a block in the cell cycle. Here we show that, compared to wild-type BAF, progeric BAF exhibits a reduced residency at sites of nuclear rupture suggestive of an impaired ability to bind to DNA. Compared to wild-type lamin-A, progeric lamin a exhibits a substantially delayed and reduced accumulation at sites of nuclear rupture. Collectively, these results begin to support the hypothesis that progeria-associated mutations in A-type lamins, BAF, and LEMD2 lead to an enhanced propensity for nuclear rupture and/or compromised nuclear rupture repair, contributing to progeria phenotypes including increased DNA damage and cellular senescence.

P2127/B374

Lipid Droplets Deform Nucleus and Cause Mislocalization of Dna Repair Factor.

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We have shown previously that high nuclear curvature imposed by pores or probes or else by small micronuclei correlates with nuclear rupture and subsequent mislocalization of multiple DNA repair factor and DNA damage. This is especially true for high rates of deformation. Lipid droplets formation in cells undergoing adipogenesis also indent nuclear lamina but if bending of the lamin filaments can cause nuclear envelope rupture, similar to one caused by a stiff probe is not clear. Here we investigate oleic acid induced lipid droplets in U2OS cells and their impact on the nucleus. Abundance of lipid droplets increased DNA repair factors mislocalization in both 2D culture and pore migration assay. Viscoelastic behavior of fat is a key to understand how lipid droplets interact physically with nucleus so here we used micropipette aspiration experiments on tissue and cellular level which revealed that fat tissue is highly

elastic, it can sustain high stresses but eventually ruptures with oil content leaking out of the cell membrane.

P2128/B375

Factors Promoting Nuclear Envelope Assembly in the Absence of Cmp7.

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In diverse organisms, the nuclear envelope (NE) undergoes dynamic restructuring that requires membrane fusion mechanisms to maintain NE integrity. Defective NE integrity is linked to aging, genome instability and cancer. Recently, the ESCRT system was found to promote membrane fusion during NE assembly and repair. ESCRT is activated at the NE when the ESCRT adapter CHMP7/Cmp7 interacts with the inner nuclear membrane protein, LEMD2/Lem2. However, in the systems studied to date, the loss of Cmp7 only partially compromises NE integrity, suggesting unknown parallel mechanisms. To identify such mechanisms, we studied postmitotic NE assembly in *S. japonicus,* an unusual fission yeast that undergoes partial mitotic NE disassembly. *S. japonicus* cells lacking Cmp7 have compromised NE integrity, but are viable in normal growth conditions. A genetic screen was performed to identify mutations that enhance the sealing of NE membranes in the absence of Cmp7. We identified gain-of-function mutations in another ESCRT adapter, Alx1, which overlaps with Cmp7 at the NE. Unexpectedly, loss-of-function mutations in Lem2 and a mutation in its interacting partner Nur1 also enhanced NE integrity of *cmp7Δ* cells. This may be explained by the fact that in the absence of Cmp7, Lem2 formed aggregates that may interfere with ESCRT-independent NE sealing. Additionally, the screen results suggest an important role for lipid composition in NE sealing. Based on these findings, we discuss models for ESCRT-independent NE sealing.

P2129/B376

Phase Separation by Membrane Protein Lem2 Controls Escrt-mediated Nuclear Envelope Reformation.

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The open mitosis strategy employed by many eukaryotes requires dramatic membrane remodeling. For proper chromosome segregation, the nuclear envelope dissociates from chromatin and retracts into the contiguous endoplasmic reticulum (ER). At mitotic exit, ER-derived membranes return to chromatin and spread to reform the nuclear envelope within approximately 12 minutes. In a series of dynamic events, the cell must coordinate spindle microtubule disassembly with nuclear envelope sealing. Here, we combine in vivo and in vitro approaches, including light and electron microscopy, to study the molecular principles of nuclear envelope reformation. Our data show how the two-pass inner nuclear membrane protein, LEM2, directs microtubule remodeling and nuclear envelope sealing via the Endosomal Sorting Complexes Required for Transport (ESCRT) pathway. LEM2's amino-terminal proline-arginine-rich low-complexity domain undergoes liquid-liquid phase separation to coat spindle microtubules. LEM2s activity is further spatially restricted to the chromatin disks by a direct binding between the LEM motif and the chromatin-associated barrier-to-autointegration factor (BAF). Once condensed, LEM2 tethers the reforming nuclear envelope the to the spindle and recruits the ESCRT-II/ESCRT-III hybrid protein CHMP7 via LEM2's carboxyterminal winged-helix domain. Together LEM2 and CHMP7 copolymerize

around microtubule bundles to form a molecular “O-ring” that promotes nuclear compartmentalization and initiates downstream ESCRT factor recruitment. These results demonstrate how multivalent interactions of a transmembrane protein - including those that mediate phase separation and coordinate localized ESCRT polymerization - enable mitotic spindle disassembly and promote membrane fusion. Consequently, defects in this pathway compromise spindle disassembly, nuclear integrity, and genome stability.

P2130/B377

The Coordinated Assembly of a Functional Nuclear Envelope After Mitosis Requires the Transmembrane Nucleoporin NPP-22/NDC-1.

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During nuclear reformation after open mitosis, nuclear pore complexes (NPCs) assemble from smaller subcomplexes onto chromatin to generate a functional nuclear envelope (NE). To initiate NPC assembly, the recruitment of the essential scaffold nucleoporins (Nup160/107 complex) by the DNA-binding nucleoporin MEL-28/ELYS is followed by recruitment of transmembrane nucleoporins and their associated membranes. In *C. elegans* embryos, MEL-28/ELYS recruits the protein phosphatase PP1c to the nuclear periphery to provide the signal that initiates nuclear assembly and growth. However, how NPC assembly coordinates with membrane recruitment during nuclear formation remains unclear. Here, we use *C. elegans* embryos to demonstrate that loss of the transmembrane nucleoporin *ndc-1*delays, but does not inhibit, the formation of a functional NE after mitosis. While the initial recruitment of ER-derived membranes, MEL-28/ELYS and scaffold nucleoporins to the reforming NE occurs normally in *ndc-1* RNAi-depleted embryos, the onset of nuclear transport is significantly delayed. Furthermore, the speed of nuclear expansion is slower in *ndc-1* RNAi-depleted embryos leading to reduced nuclear size. These embryos contain lowerlevels of MEL28/ELYS and the essential scaffold nucleoporins (Nup160, Nup133, and Nup85). These data suggest that NDC-1 controls the timely maturation of nascent NPCs to direct NE formation and growth by stabilizing scaffold nucleoporins. Consistent with this idea, reduced nuclear size, but not the delayed onset of nuclear transport, in *ndc-1* RNAi-depleted embryos can be partially rescued by deletion of *cnep-1 to* increase nuclear membrane incorporation after mitosis. Thus, NDC-1 is required for the formation of transport competent NPCs after mitosis, likely through its role in stabilizing scaffold nucleoporins rather than a role in membrane recruitment to initiate NPC assembly. We suggest that NDC-1 controls the concentration of scaffold nucleoporins, which allows for the coordinated maturation of NPCs to drive assembly and growth of a functional NE during nuclear reformation.

P2131/B378

Can Nuclear Envelope Transmembrane Proteins Tether Pluripotency.

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A vast majority of Nuclear Envelope Transmembrane Proteins (NETs) are tissue-specific. These tissue-specific NETs maintain tissue-specific 3D genome organization. Furthermore, interfering with the expression of NETs impairs differentiation. TMEM 120A and B, enriched in adipose tissue, are important for adipogenesis while NET39, TMEM38A and WFS1, enriched in muscles, are important for myotube formation. Although a functional basis for relocation of genes during lineage specification has been reported, the components of the nuclear envelope that might have a role in orchestrating this are

unclear. We hypothesize that as cells progress from their pluripotent state through differentiation into defined lineages, they acquire different NET signatures which might bring about the coordinated changes in genome organization. During exit from pluripotency of mouse embryonic stem cells, we found that certain genetic loci containing pluripotency genes like Triml1, Zfp42 and Nanog show rapid movement from the nuclear interior towards the periphery, suggesting that reorganisation of the genome is among the very first of events occurring during lineage specification. Ectopic expression of some tissue-specific NETs in these cells leads to a reduction in pluripotency as measured by morphological differences and loss in alkaline phosphatase activity. Finally, the loss of pluripotency is coupled with genome organization changes observed during exit from pluripotency. We are currently investigating which NETs and/or their post translational modifications might drive the reorganization of the genome during exit from pluripotency and whether ectopic expression of tissue-specific NETs can aid in lineage commitment.

P2132/B379

Understanding the Mechanism Behind Membrane Fusion during Pronuclear Meeting in *C. Elegans* Embryo.

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In a just fertilized *C. elegans* embryo, to restore ploidy, the nuclear envelope (NE) of the haploid genomes (a.k.a pronuclei) must disassemble to allow mixing of the two parental genomes. Interestingly, while proteins associated with the NE disassemble, the pronuclear membranes persist after pronuclear meeting. It is still unclear how these membranes are breached to allow parental chromosome mixing. To understand this, we have used Focused-Ion Beam Scanning Electron Microscopy (FIB-SEM) of nuclear membranes of early 1-cell embryos. The FIB-SEM data revealed the presence of unique membrane junctions, where the two pronuclei fuse. These membrane junctions are not observed at the pronuclear interface in *plk-1*^{ts} embryos, which are defective in parental chromosomal mixing. To address the mechanism behind junction formation, we explored the role of the ER fusion machinery, and in particular the role of ATLN-1 (human atlastin1 homolog). Dilution RNAi of atlastin showed, at low penetrance, paired nuclei formation, an indication of defective pronuclear fusion. To better control the extent of ATLN-1 downregulation, I used the auxin-induced degradation system for the temporal degradation of *atln-1* in early embryos. Auxin-mediated degradation of a degron-tagged *atln-1* led to paired nuclei formation in nearly 100% of the observed 1-cell embryos, suggesting its role in pronuclear membrane fusion. Further, GFP tagged endogenous ATLN-1 showed that it localizes to the ER, NE, and centrosomes in *C. elegans* embryos. We found that ATLN-1 is also present at the interface of the two pronuclear membranes. Together, these results suggest that atlastin-mediated membrane fusion is required for pronuclear membrane permeabilization, allowing for parental chromosome mixing. Surprisingly, ATLN-1 is only required for LMN-1 (*C. elegans* lamin) disassembly at the metaphase, not for NPP-1 (a nuclear pore complex protein). My future goal is to do FIB-SEM to examine the membranes at the interface of the pronuclear membranes in the *atln-1* depleted early stage embryos.

P2133/B380

Maternal and Paternal Genome Mixing in the *C. Elegans* Zygote Involves Stepwise Pronuclear Fusion and Fenestration of Pronuclear Membranes.

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In a fertilized embryo, maternal and paternal genome that are initially present in two separate pronuclei must mix in a process that requires nuclear envelope breakdown (NEBD). NEBD in the zygote is poorly understood, especially in mammals where visualizing the process is challenging. It is assumed that once the parental pronuclei are in close apposition NEBD is initiated. In *C. elegans,* once the maternal and paternal pronuclei meet, a gap is formed in the juxtaposed membranes, close to the metaphase-aligned chromosomes. This membrane gap is critical for parental genome mixing. Recently, we reported that in *C. elegans,* partial depletion of the conserved Polo-like kinase, PLK-1, leads to the formation of paired-nuclei with separated parental genomes due to a failure in gap formation in the NEs between the two pronuclei. The structure of NE membrane gap and the signals that promote its formation are unknown. To understand this process we used two techniques, live-cell fluorescence microscopy and electron microscopy, specifically Focused Ion Beam - Scanning Electron Microscopy (FIB-SEM). Live-cell fluorescence images revealed that a stable interface is formed between two juxtaposed pronuclear membranes, and that at metaphase the interface retains both inner and outer nuclear membrane proteins. Zygotes were collected at different stages of mitosis and subjected to high-pressure freezing prior to FIB-SEM. Interface membranes reconstructed from SEM images taken at 9 nm resolution revealed that the interface is composed of only two membranes that are flanked by a novel type of membrane junction, a 3-way sheet junction, present between the opposing pronuclei at metaphase. Three-way sheet junctions reduced the number of pronuclear membranes in the interface from four parental membranes to two. An alysis of multiple prometaphase- and metaphase-stage embryos by FIB-SEM uncovered a second type of membrane junction between the outer nuclear membranes of the two opposing pronuclei. In contrast, *plk-1* mutant zygotes were devoid of junctions, indicating that junction formation is a prerequisite for mixing of parental genomes. We hypothesized that proteins involved in endoplasmic reticulum (ER) remodeling may be involved in junction formation. Our preliminary data suggest that Atlastin, a protein known for its role in ER-ER homotypic fusion, is required for the process.

P2134/B381

Autonomous Asymmetric Nuclear Division Generates Daughter Nuclei with Distinct Identities during Neuroblast Divisions in the Fly.

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Asymmetric cell division generates cellular diversity through unequal partitioning of cell fate determinants. Fly neural stem cells rely on asymmetric cell division to generate a self-renewing stem cell and a smaller differentiating sibling cell. Although the role of the cortical polarity on cell fate has been well documented, the contribution of the nuclear division in this context remains to be explored. Here, using live cell imaging of asymmetric divisions in intact and dissociated brains, together with electron microscopy, mutant analysis and drug treatments, I show that the nucleus compartment retains its identity during division. Second, I show that nuclear division does not require cell division, but is rendered asymmetric by the asymmetric division furrow. Third, I find that the generation of two

daughter nuclei with a distinct size and composition occurs in two-steps - establishment and growth. Finally, I show how the coupling of nuclear division with the release of cortical cell fate determinants help to establish two different nuclear identities that contribute to generation of distinct cell fates during a neuroblast division.

P2135/B382

The Escrt-iii Complex Mediates the Reorganization of the Meiotic Nuclear Envelope in Budding Yeast.

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The nuclear envelope separates the nucleoplasm from the cytoplasm in eukaryotic cells. Embedded into the nuclear envelope, the nuclear pore complexes (NPCs) are large multi-subunit structures that form an aqueous channel, allowing for passage between the nucleus and cytoplasm. Unlike higher eukaryotes, budding yeast undergoes closed mitosis and meiosis with little to no detectable nuclear envelope breakdown. In higher eukaryotes, nuclear envelope breakdown serves as part of a mechanism to rid the nucleus of unwanted material or senescence factors, such as old or damaged NPCs. In mitosis, budding yeast retains these potentially harmful factors in the mother cell through asymmetric cell division, with the daughter cell gaining a full replicative lifespan. Cleavage of the membrane at the bud neck by the different Endosomal Sorting Complexes Required for Transport (ESCRT) complexes is required for this asymmetric division and results in the mother cell having a finite replicative lifespan. However in budding yeast meiosis, the age of the mother cell is irrelevant, four haploid gametes are produced without any of the mother's unwanted material or senescence factors. Meiosis therefore serves as a reprogramming and/or rejuvenation process. Recent studies have found this rejuvenation depends on the meiotic reorganization of the nuclear envelope, which clusters the senescence factors, including oxidatively damaged protein aggregates, rDNA circles and NPCs, during meiosis II into a centralized cellular compartment, termed the fifth compartment, distinguished from the four gametes formed after meiosis. However, the mechanism for this nuclear reorganization has yet to be determined.Here we show that the clustering of NPC components in anaphase II occurs as an active sequestration process mediated by Chm7 and Heh1, components associated with the ESCRT-III complex. Chm7-Heh1 has been shown to function in mitotic yeast cells to seal the nuclear envelope upon defective NPC formation, and also to serve as a constriction factor at the bud neck of dividing cells. In meiosis, these ESCRT-III components are required for the constriction of old NPCs to the fifth compartment. We propose that the sequestration of senescence factors, such as old NPCs, is mediated by the ESCRT-III complex, enabling these unwanted or damaged components to be excluded from rejuvenated gametes. Our work may provide a mechanistic understanding of nuclear envelope reorganization and cell rejuvenation.

Nucleocytoplasmic Transport

P2136/B383

Composition and Properties of XPO1-dependent Nuclear Export Signals.

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Exportin 1 (XPO1, also known as Crm1) mediates one of the most versatile protein transport pathways of the eukaryotic cells. It transports hundreds of different proteins and protein complexes from the nucleus to the cytoplasm. XPO1 is able to recognize such a vast and diverse set of cargos thanks to the ability to bind a peptide motif called a nuclear export signal (NES). Despite more than two decades of

extensive research, the features that contribute to a NES's ability to recruit XPO1 remain poorly understood. To gain comprehensive understanding of the composition rules for the NESs, we employ phage display combined with high-throughput DNA sequencing. Such approach allows us to cover the entire sequence space for the peptides of appropriate length and to analyze contributions of any residue at each position of the motif. We discovered numerous features that affect, positively or negatively, the ability of a peptide to bind to XPO1. We confirm the discovered contributions of various residues at specific positions in *vivo*. Armed with the dataset covering the entire sequence space for a NES motif, we rationally design peptides to have the highest possible propensity for binding XPO1. Indeed, we confirm that the interactions of the designed peptides with XPO1 cannot be disrupted even using a competitive suicide inhibitor of XPO1. We characterize the interaction of one of these peptides with XPO1 using X-ray crystallography. We see numerous interactions from residues at different positions contributing to an unprecedentedly strong binding to XPO1. We show that the designed peptides act as XPO1 inhibitors themselves in *vivo*. Finally, we propose an inclusive way of sequence-based NESs prediction. By assessing a clearly defined contribution of each residue at each position of a potential NES, we can provide an accurate prediction of XPO1 binding propensity for any sequence of residues. Ability to predict potential NES for any protein based on its sequence is useful in studying new proteins and characterizing their transport and logistics. XPO1 is one of the major drug discovery targets for treatment of different cancer types and is involved in various viral infections including HIV-1. Thus, we expect that our data might prove useful in medical research.

P2137/B384

Nuclear Pore Complex Scaffold Experiences Conformational Changes in *Vivo in* Response to Transport State.

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Recent breakthroughs in structural studies have provided a wealth of information about the architecture of the nuclear pore complex (NPC). However, measuring the dynamics of nucleoporins (Nups) in *vivo* remains highly challenging. By using polarized-total internal reflection fluorescence microscopy (pol-TIRFM), we have measured the orientations and dynamics of Nup-mEGFP fusion proteins in *vivo.* Our measurements have shown that components of the NPC scaffold assume distinct conformational states under various transport conditions. We developed imaging tools that allow us to visualize protein dynamics within diffraction-limited puncta in *vivo. By* rapidly switching the polarization of our excitation light between two orthogonal polarizations, we can measure the orientation of mEGFP. We conjugated mEGFP to several Nups via rigid alpha helical linkers and used the orientation of the mEGFP to report on the orientation of the Nups of interest. Using this technique, we measured the orientations of domains within NUP133, NUP54, NUP93, and NUP58. After confirming that the mEGFP reports on the orientation of our Nup of interest, we then used these conformational sensors to measure the orientations and dynamics of Nups within different parts of the NPC. Using CRISPR/Cas9, we replaced all endogenous copies of the Nups with our mEGFP-Nup fusion proteins and were able to confirm that the NPCs were functional in *vivo*. Under conditions of cargo depletion, the NPC assumed one distinct conformation. Under conditions of excess cargo, the NPC was driven into an alternate state. We modulated the transport state in a number of ways: starving cells, using drugs to block export, using dominant negative proteins, and finally by introducing nuclear transport factors directly to the nuclear periphery by permeabilizing the plasma membrane while leaving the nucleus intact. Under each of these perturbations, we found that most regions of the NPC do not shift their orientation in response to cargo,

but specific domains of the inner ring complex are reorganized with altered in *vivo* transport state. Our results suggest that the NPC experiences a large-scale conformational change in response to cargo translocation. In addition, our results demonstrate how fluorescence polarization microscopy is a powerful tool for monitoring the organization and dynamics of proteins in *vivo and* in *vitro*.

P2138/B385

Uncovering the Role of O-GlcNAc Modification in Nuclear Pore Complexes by Live-cell Measurement of Nuclear Transport Rates.

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The nuclear pore complexes selectively facilitate the transport of proteins with nuclear localization or export signals (NLS/NES), while limiting the traffic of other macromolecules. The nuclear pore components are remarkably rich in Ser/Thr-linked monosaccharides, O-linked β-N-acetyl glucosamine (O-GlcNAc), particularly within the intrinsically disordered FG-repeat regions. The O-GlcNAc-rich FG-repeats are known to be indispensable for the signal-dependent nuclear transport. Previous study showed that O-GlcNAc modification modifies the permeability and structure of in vitro-formed hydrogel derived from FG domains. However, the role of O-GlcNAc of nuclear pore complexes in nuclear transport is still unclear. Here we develop an imaging-based technique that quantitatively measures the rates of signal-dependent nuclear import and export in live cells. Using the technique, we found that the nuclear transport rate in cells is significantly altered after perturbations of O-GlcNAc transferase (OGT) or O-GlcNAcase (OGA), a single pair of enzymes that controls the O-GlcNAc modification. The change is bidirectional in a manner correlated with the level of O-GlcNAc modifications in the nuclear pore complexes. Interestingly, the nuclear import rate increases more dramatically with elevated O-GlcNAc level than the nuclear export rate does. We are further investigating the mechanism underlying the differential changes in nuclear import and export rates upon O-GlcNAc perturbations and its implications in the control of compartmentalization of nucleocytoplasmic shuttling proteins, nutrition sensing, and cellular stress response.

P2139/B386

The Mechanosensitivity of Nucleocytoplasmic Transport Is Governed by Increased Active Transport Both into and Out of the Nucleus.

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Mechanical force drives fundamental processes in health and disease, and regulate transcription. Increasing evidence shows that the cell nucleus responds to force, for instance by increasing nuclear import of the transcriptional regulator YAP [1]. However, whether nucleocytoplasmic transport is a mechanosensitive process, the mechanism involved, its applicability to different cargoes, and its molecular specificity remain unknown. Here we show that nuclear force promotes active versus passive transport in both directions, making nucleocytoplasmic transport mechanosensitive. We carried out static and dynamic measurements on proteins of different molecular weights (thereby regulating diffusivity through nuclear pores) containing nuclear import signals (NLS) or nuclear export signals (NES) of different strengths. We observe that both active import and export increase with nuclear force, either applied directly (via AFM) or indirectly (by plating cells on substrates of different rigidities). Further, we show that the force-dependent steady state localization of the protein depends on a balance between

active transport and passive diffusion. Finally, we show the applicability of this mechanism to different transcriptional regulators that enter or exit the nucleus with force. Our study shows a general mechanism by which cargoes specifically respond to nuclear force by entering or exiting the nucleus, which the cell uses as a control of mechanosensitive transcription. [1] Elosegui-Artola et al., Force Triggers YAP Nuclear Entry by Regulating Transport across Nuclear Pores, Cell (2017), https://doi.org/10.1016/j.cell.2017.10.008

P2140/B387

Nuclear Size Is Determined by Its Content, Not Its Envelope.

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Nuclear size regulation is a fundamental biological process conserved from single-celled eukaryotic organisms to human cells. Within a given cell type, nuclear size scales with cell size to produce a fairly constant ratio. The dysregulation of the nucleus-to-cell volume ratio (N:C volume ratio), is a hallmark for many cancerous cells. However, the exact mechanisms regulating nuclear size are unknown. Previous work in Xenopus and fission yeast suggests nucleocytoplasmic transport as an important process regulating nuclear size: increasing nuclear protein import and inhibiting its export enlarges nuclear size. However, this did not seem to be the case in budding yeast. Nonetheless, given a previous finding from our lab that the nuclear envelope does not determine nuclear size, we revisited the possibility that the amount of protein in the nucleus regulates its size. To test this, we inhibited the well-conserved general nuclear export protein Crm1 using Leptomycin B (LMB). Importantly, inhibition of export led to an increase in nuclear volume and a concomitant increase in the N:C volume ratio. These data suggest that nuclear size is dependent on its content. We next asked if this increase in nuclear volume relies on continued nuclear membrane synthesis. We inhibited fatty acid synthesis (a phospholipid precursor), which is known to block nuclear growth in other expansion phenotypes. Blocking membrane synthesis had no significant effect on the increase in nuclear growth seen with LMB treatment, suggesting that in the presence of LMB, nuclear expansion is independent of lipid availability. These data raise several questions: Is nuclear size determined by the total amount of nuclear protein or by the accumulation of specific proteins? Do cells normally control nuclear size by regulating the amount of protein in the nucleus, or is it a passive consequence of the amount of nuclear protein synthesized by the cell? How do the cells accommodate an expanding nucleus without available lipids? Our efforts toward answering these questions will be discussed.

P2141/B388

Formin-mediated Regulation of Nuclear Morphology in Actin-intact *Xenopus* Extracts.

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Xenopus egg extracts are a useful system to study the regulation of nuclear morphology because the levels and activities of extract components are easily manipulated. In order to form nuclei in egg extracts, demembranated sperm is added as a DNA source, and cycloheximide blocks progression into mitosis. Generally, extract is also supplemented with cytochalasin to depolymerize F-actin and prevent extract gelation and contraction. Nuclei assembled in cytochalasin-treated egg extracts tend to be roughly spherical. Because F-actin has been shown to affect nuclear morphology and growth in other systems, we tested if dynamic F-actin affects in vitro nuclear morphology using actin-intact extracts lacking cytochalasin. Indeed, we observed that nuclei in actin-intact extracts were often blebbed with a

bilobed morphology. Interestingly, the larger nuclear lobe usually lacked Hoechst-staining DNA and exhibited higher actin signal, fewer nuclear pores, and reduced lamin staining. By co-staining for F-actin and nuclear pores, we identified a strong intranuclear actin signal juxtaposed to the nuclear envelope. Through various inhibition experiments, we determined that the bilobed nuclear morphology and enrichment of F-actin at the nuclear envelope were dependent on formins, that nucleate unbranched F-actin filaments, but not on myosin or Arp2/3. These data suggest that formin-mediated intranuclear F-actin dynamics underlie the bilobed nuclear morphology observed in actin-intact extracts. We are continuing to test this hypothesis in extracts and in vivo in *Xenopus* embryos.

P2142/B389

Macronuclear Shape Change in the Giant Ciliate, *Stentor Coeruleus*.

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A fundamental question relating to cellular spatial patterning is how nuclei are shaped within cells. The giant ciliate *Stentor coeruleus* provides a unique opportunity to investigate the mechanisms behind nuclear shape, because *Stentor* takes this aspect of the nucleus to the extreme: *Stentor* can reach 1 mm in length, and it possesses a correspondingly gigantic macronucleus shaped like a string of spherical beads. During cell division, the macronucleus dramatically changes shape before dividing amitotically into the two daughter cells. The macronucleus condenses into single sphere, extends, and renodulates in 2-3 hours near the end of cell division. We can experimentally induce this macronuclear shape change by causing *Stentor* to regenerate its oral apparatus. The morphological events during regeneration are similar to cell division, including the cycle of shape changes performed by the macronucleus. It is unclear how this extreme macronuclear shape change is regulated. While microsurgical and electron microscopy studies addressed this question in the past, we have had virtually no molecular insight into this feat of subcellular morphogenesis. Our first molecular foothold into this question comes from an RNAi screen of genes differentially expressed during regeneration in *Stentor*. We found that a homolog of the nuclear exportin gene CSE1 was among the top differentially expressed genes during this process. In yeast, CSE1 is required to export importin-alpha from the nucleus. In humans it is reported that CSE1 also co-localizes with microtubules. We tested the function of this gene in *Stentor* using RNAi. Knockdown of CSE1 results in *Stentor* with clumped and irregularly shaped macronuclear nodes, suggesting CSE1 plays a role in elongation of the macronucleus. Immunofluorescence of CSE1 in *Stentor* results in punctate staining in the cytoplasm with no staining in the macronucleus during the early stages of cell division and regeneration. When the macronucleus is condensed, CSE1 puncta appear in the interior of the macronucleus. Previous electron microscopy studies have shown that the elongating macronucleus is surrounded by a transient microtubule mesh, and contains nuclear envelope-bound channels full of microtubules. Given CSE1's known roles in other systems, we are currently investigating whether CSE1 facilitates macronuclear elongation by interacting with these transient microtubule structures or by another mechanism involving nuclear transport.

P2143/B390

Pcid2 Influences Brca1/bard1 Localization and Centrosome Duplication through It'S Functions in Nuclear Protein and Mrna Export.

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Nuclear protein export has an emerging role in the regulation of centrosome duplication, a function that involves interaction of centrosomal proteins with the Crm1 nuclear export factor. The protein PCID2, first identified as an mRNA export factor, also functions in Crm1 mediated protein export, with PCID2 siRNA knockdown slowing the rate of export. In addition, PCID2 has been localized to the centrosome. Despite the fact that PCID2 is poised to be a cell cycle regulator through potential combination of it's centrosomal and nuclear export roles, the function of PCID2 at these locations has not been well characterized. We sought to elucidate a potential link between these roles of by exploring PCID2's involvement in the export of particular cargo known to transit between these the nucleus and centrosome. Our studies focus on BRCA1 and BARD1 as potential PCID2 nuclear to centrosomal cargo, as these DNA damage repair proteins transit from the nucleus to the centrosome in a Crm1 dependent manner, particularly in times of cellular stress. Knockdown of PCID2 caused a 10% increase in the nuclear retention of BRCA1, with a concomitant 40-fold decrease in centrosomes exhibiting BRCA1 localization. These results suggest a role for PCID2 in the export of the BRCA1 protein from the nucleus and in it's subsequent localization to the centrosome. Conversely, BARD1 nuclear levels decreased by nearly 20% in the absence of PCID2, which was accompanied by a 40-fold decrease in BARD1 positive centrosomes. These effects appear to stem from a 50% drop in BARD1 protein levels in PCID2 knockdown cells, suggesting a potential role for PCID2 in the export of BARD1 mRNA rather than protein, with failure to export contributing to loss in protein expression. Additionally, altered transport of BRCA1/BARD1 due to PCID2 knockdown had an unexpected effect on centrosome duplication. Rather than the increase in centrosome number seen with BRCA1/BARD1 knockdown, loss of PCID2 led to a four-fold reduction in cells with an aberrant number of centrosomes (3 or more). Furthermore, treatment with inducers of excess centrosome duplication led to an increase in PCID2 positive centrosomes. Together these results suggest a positive role for PCID2 in centrosome duplication. This unexpected outcome is potentially due to the use of non-breast cancer cells. Our ongoing studies include characterizing the function of PCID2 in breast cancer cells, and it's relationship with BRCA1 and BARD1 in that environment as well as investigating the role of PCID2 in BARD1 mRNA export. Determining how PCID2 influences the cellular localization of the BRCA1/BARD1 complex will help to elucidate the mechanism of PCID2's effect on centrosome duplication and therefore determine it's potential impacts on cell cycle regulation and tumor development.

P2144/B391

How Cellular Stress Alters the Subcellular Localization of Oncomodulin.

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Oncomodulin (OCM) is an EF-hand calcium-binding protein expressed in outer hair cells (OHCs) of the cochlea, macrophages and neutrophils, and a subset of placental cells. Despite its unique expression, the mechanism by which OCM influences Ca^{2+}-signaling pathways, and how it may differ from its more ubiquitous alpha isoform (α-parvalbumin, αPV), remains elusive. Deletion of OCM in C57Bl/6 mice produces no observable phenotype at 1 month. However, by 4 months, these mice are essentially deaf, as measured by auditory brainstem response (ABR) threshold shifts and loss of distortion product

otoacoustic emissions (DPOAEs). OCM mutants exhibit OHC loss and distinct morphological changes. These observations suggest that OCM promotes the survival of OHCs throughout adulthood. Interestingly, we have observed nuclear expression of OCM in OHCs from wild-type aged mice. Therefore, we hypothesize that OCM has a unique function within OHCs and can promote cell survival during stress conditions. In order to understand the mechanism behind OCM's ability to promote cell survival, we used in *vitro* analyses. Calcium-related cellular stressors were used to induce nuclear localization of exogenous GFP-tagged OCM. DS16570511, a small-molecule mitochondrial calcium uniporter (MCU) inhibitor, and thapsigargin, an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) were administered to HeLa cells transiently expressing OCM-GFP. 24 hours after treatment with these cellular stressors, we observed significantly higher amounts of OCM in the nucleus than in the cytoplasm compared to non-treated controls. In contrast, αPV, which shares over 53% sequence homology to OCM, showed a clear preference for cytoplasmic localization under the same cell stressors. Additionally, both OCM and αPV plasmids significantly increased the time course of transient Ca^{2+} signaling after stimulating HeLa cells with ATP. These results demonstrate that although OCM can buffer calcium similarly to αPV, OCM may respond to cellular stressors differently than other CaBPs and reveals the first evidence of a potential mechanism of OCM function in OHCs.

P2145/B392

Insights into the Role of Nup358 in Mirna Pathway through Its Interaction with an Rna Binding Protein.

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Nuclear envelope (NE) is a double layered membrane that encircles the nucleus, and it consists of nuclear pore complex (NPCs) that regulates transport of macromolecules between the nucleus and the cytoplasm. Each mammalian NPC contains around 30 different nucleoporins in multiple copies. Nup358 is a 358kDa nucleoporin localized to the cytoplasmic fibrils of the NPC and also to the cytoplasmic annulate lamellae (AL), subdomain of Endoplasmic reticulum (ER). Previous findings from our lab have shown association of these AL structures with two messenger ribonucleoprotein (mRNP) granules: processing bodies (P bodies) and stress granules (SGs). Further investigation revealed that Nup358 is required for microRNA (miRNA)-mediated post-transcriptional gene silencing. It has been observed that Nup358 is essential for association of target mRNA with miRNA induced silencing complex (miRISC). Moreover, it was found that Nup358 physically interacts with the components of miRISC such as AGO and GW182 proteins. Further our efforts defined the N-terminal 993 amino acids to be the minimum region within Nup358 required for its function in miRNA pathway. Extreme N-terminal region of Nup358 has an alpha helical domain harbouring three tetratricopeptide repeats (TPRs), which was previously reported to bind to single stranded RNA. Our studies using luciferase-based miRNA reporter assays revealed that the TPR region is important for miRNA pathway. We wished to investigate if mRNA export machinery coordinates with Nup358 to couple the mRNA to miRISC. We found that Nup358 interacts with DDX19 (human orthologue of yeast Dbp5), an RNA helicase involved in mRNA export by remodelling the mRNPs at the cytoplasmic side of the NPC. We also found that DDX19 interacts with the N-terminal region of Nup358. Furthermore, depletion of DDX19 impaired the miRNA pathway. These results are consistent with the hypothesis that DDX19 cooperates with Nup358 in coupling the exported target mRNA with miRISC. The studies thus will provide further insights into the mechanism by which mRNA export is linked to the miRNA-mediated suppression of mRNA translation.

P2146/B393

Dbp5 Interacts with Mex67 and Nab2 at the Nuclear Pore Complex for Specific Release during Mrnp Remodeling.

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Transcripts are coated with RNA binding proteins (RBPs) that dictate processes such as messenger (m) RNA localization, translation, and degradation. As mRNA proceeds through its lifecycle, the complement of associated RBPs is selectively exchanged to indicate successful processing and allow subsequent steps to take place. The major class of molecules responsible for this RBP exchange are DEAD-box proteins, which induce local ATP-dependent RNA structural changes and RBP release. However, in many cases, the mechanism for DEAD-box protein-mediated removal of specific RBPs is not fully defined. To explore this selectivity, we are studying the nuclear mRNA export process. In *Saccharomyces cerevisiae,* the mRNA export receptor Mex67 is recruited to mature nuclear transcripts allowing export of mRNA through the nuclear pore complex (NPC) to the cytoplasm. Mex67 binds transcripts through adaptor proteins such as the poly-A binding protein Nab2. When a transcript reaches the cytoplasmic face of the NPC, the DEAD-box protein Dbp5 triggers selective release of Nab2 and Mex67 from the mRNA. This imposes directionality to mRNA export while allowing the remaining nuclear-associated RBPs to function in the cytoplasm. It is unknown how Dbp5 specifically targets Nab2 and Mex67 for removal from exporting transcripts. Furthermore, in addition to its mRNA export roles at the NPC, Dbp5 has proposed functions throughout the mRNA lifecycle during cytoplasmic translation and in the nucleus, and it is unclear whether these diverse functions are interdependent. Here we have found that Dbp5 binds Mex67 and Nab2 via protein-protein interactions. Using a Split-GFP-based approach, these interactions were only detectable at the NPC and not prior to export, indicating that specificity is determined upon mRNA export. To test whether Dbp5 action at the NPC is sufficient for mRNA export, Dbp5 was anchored at the NPC cytoplasmic face to Nup159. This Nup159-Dbp5 fusion was sufficient for cell viability. Thus, for mRNA export, Dbp5 only needs to bind mRNA at the NPC. Therefore, Dbp5 has separable functions in a cell, and specific interactions direct specificity in these activities.

P2147/B394

Structural Basis for Influenza Virus Ns1 Protein Block of Mrna Nuclear Export.

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Influenza viruses antagonize key immune defence mechanisms via the virulence factor non-structural protein 1 (NS1). A key mechanism of virulence by NS1 is blocking nuclear export of host messenger RNAs, including those encoding immune factors; however, the direct cellular target of NS1 and the mechanism of host mRNA export inhibition are not known. Here, we identify the target of NS1 as the mRNA export receptor complex, nuclear RNA export factor 1-nuclear transport factor 2-related export protein 1 (NXF1-NXT1), which is the principal receptor mediating docking and translocation of mRNAs through the nuclear pore complex via interactions with nucleoporins. We determined the crystal structure of NS1 in complex with NXF1-NXT1 at 3.8 Å resolution. The structure reveals that NS1 prevents binding of NXF1-NXT1 to nucleoporins, thereby inhibiting mRNA export through the nuclear pore complex into the cytoplasm for translation. We demonstrate that a mutant influenza virus deficient in

binding NXF1-NXT1 does not block host mRNA export and is attenuated. This attenuation is marked by the release of mRNAs encoding immune factors from the nucleus. In sum, our study uncovers the molecular basis of a major nuclear function of influenza NS1 protein that causes potent blockage of host gene expression and contributes to inhibition of host immunity.

P2148/B395

Transcriptional Regulation of Ribosomal Proteins by Karyopherin Kap114p-mediated Trans-repressionunder Saline Stress.

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Transcriptional regulation of ribosomal proteins by Karyopherin Kap114p-mediated trans-repression under saline stress Abstract Gene expression is controlled by nuclear accessibility of transcription factors that are co-regulated by transport receptors and competitive regulators. Whether nuclear import receptors have activities beyond their transport function in transcription factor-related regulatory networks has never been resolved. Kap114p, one member of the karyopherin-βs (Kap-βs) that mediate nuclear import of yeast TATA-binding protein (yTBP), exhibits a yTBP-binding affinity three orders of magnitude higher than its counterparts. Our crystal structure of Kap114p reveals an extensively negatively-charged concave surface, accounting for high-affinity basic- protein binding. Moreover, we biochemically demonstrate that Kap114p suppresses binding of yTBP with DNA and transcription factors, facilitated by two intra-HEAT-repeat inserts. Furthermore, *Kap114* knockout in yeast leads to a high-salt growth defect, with transcriptome analyses revealing that Kap114p modulates expression of genes associated with ribosomal biogenesis and protein translation by repressing yTBP binding to target promoters. Hence, apart from mediating nuclear import, Kap114p inhibits yTBP activity via a trans-repression mechanism, down-regulating protein synthesis and facilitating yeast growth under high- salinity stress.

P2149/B396

Importins Regulating Histone Dynamics in Drosophila.

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Following synthesis in the cytoplasm, histones are handled by an elaborate network of chaperones before being incorporated into chromatin. Histones are typically rapidly imported in the nucleus and, hence the earliest cytoplasmic steps have not been very well elucidated. Here we take advantage of Drosophila oogenesis in which histone synthesis and nuclear import are separated in time. Massive amounts of the histone variant H2Av are synthesized in late-stage oocytes and dynamically stored on lipid droplets (LDs), cytoplasmic fat storage organelles. H2Av constantly exchanges between LDs, and only after fertilization are these H2Av stores gradually used to package the newly synthesized DNA in the nuclei of the embryo. In ongoing proteomic experiments, we are characterizing proteins that bind to H2Av in the cytoplasm. Because recent reports have implicated members of the importin family as cytoplasmic histone partners, we are also testing the role of specific importins in regulating H2Av dynamics. Using H2Av-Dendra and Fluorescence Recovery After Photobleaching, we find that H2Av

exchange between LDs is almost abolished in oocytes lacking Importin-9/Ranbp9, suggesting that this importin mediates H2Av transport through the cytoplasm. Using proteomic analysis, we also identified another importin, Importin α2 (Impα2), as an LD-associated protein in Drosophila embryos. Intriguingly, Impα2 is largely cytoplasmic in early-stage embryos but becomes a major LD protein by the cellularization stage. This relocalization correlates with a major transition in H2Av dynamics from rapid exchange between LDs to static binding. To determine whether these two processes are functionally linked, we are identifying how Impα2 binds to LDs. This binding is likely via the LD protein Jabba, as Impα2 recruitment to LDs is abolished in *Jabba* mutants and Impα2 and Jabba physically interact in cultured cells, as assessed by luciferase complementation assay. Jabba is also known to anchor H2Av to LDs, but H2Av and Impα2 binding are separable. We have generated a Jabba construct that specifically lacks the ability to interact with Impα2 and will determine if flies expressing this construct display altered histone dynamics. In summary, we have identified two different importins as candidate regulators of cytoplasmic histone H2Av.

P2150/B397

Importin β/7 Promotes Nuclear Import of the RNA-binding Protein FUS and Prevents Its Phase Separation.

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that is characterized by nuclear loss and cytoplasmic aggregation of RNA-binding proteins such as FUS (fused in sarcoma). A key factor in disease pathology is defective nucleocytoplasmic transport. ALS-associated mutations in the nuclear localization signal of FUS have been shown to reduce binding to its nuclear import receptor transportin and impair FUS import. Recently, transportin was reported to not only function as a FUS import receptor but also as a molecular chaperone to prevent and even reverse aberrant phase separation of FUS. Using transport assays in permeabilized cells, we demonstrate that nuclear import of FUS is not only mediated by transportin but also by the importin β/7 heterodimer. Binding assays suggest that FUS binds directly to several nuclear import receptors in a RanGTP-sensitive manner. Similar to transportin, importin β/7 functions as a chaperone for FUS and keeps it in solution. Taken together, our results suggest that nuclear import and chaperoning of FUS is not only mediated by transportin but also by importin β/7 and possibly other nuclear transport receptors.

P2151/B398

Heterogeneous Nuclear Ribonucleoprotein A2B1 Regulates and Is Regulated by Cellular Copper Status.

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Copper (Cu) is an essential cofactor of enzymes involved in numerous cellular processes, such as respiration, antioxidant defense, neurotransmitter production, and myelination. Cu misbalance is implicated in several human diseases, and as such, Cu homeostasis is tightly regulated by a network of Cu-binding and -transporting proteins. In an animal model of Wilson disease, a genetic disorder of Cu accumulation, Cu elevation triggers the nucleocytoplasmic redistribution of several RNA binding proteins, including heterogeneous nuclear ribonucleoprotein (hnRNP) A2B1. To better understand the interface between Cu homeostasis and RNA processing and trafficking, we investigated the relationship between Cu and hnRNP A2B1. hnRNP A2B1 has four isoforms generated by the alternative splicing of exons 2 and 9, and these isoforms can be categorized into two groups: the exon 2-containg B isoforms

and the exon 2-lacking a isoforms. We found that small interfering (si)RNA-mediated knockdown of all four hnRNP A2B1 isoforms in HeLa cells resulted in a significant and specific decrease in cellular Cu content. This was associated with an increase in both mRNA and protein levels of the Cu-transporter ATP7A. Knockdown of only the B isoforms was sufficient to increase the abundance of both ATP7A mRNA and protein, suggesting that these isoforms are the ones mainly responsible for the regulation of ATP7A. Furthermore, we observed rapid, concentration-dependent redistribution of hnRNP A2B1 from the nucleus to distinct cytoplasmic puncta that was not reversed by treatment with Cu chelators. These puncta do not stain positive for any markers of classic cytoplasmic granules, suggesting that this redistribution may be a specific response to elevated Cu. Knockdown of the B isoforms resulted in a lack of hnRNP A2B1 redistribution in response to Cu, suggesting that these isoforms are mainly responsible for this effect. These results show that the cellular properties of hnRNP A2B1 are modulated by Cu and suggest that one or more hnRNP A2B1 isoforms play a role in the regulation of ATP7A RNA involved in cellular Cu balance. These findings provide a basis for understanding how Cu homeostasis is regulated on the RNA-level as well as shed light onto a potential role for Cu in RNA metabolism.

P2152/B399

Pqbp1 Facilitates the Nuclear Import of Splicing Factor Txnl4a.

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Renpenning syndrome is a group of X-linked intellectual disability (XLID) syndromes which caused by mutations of human polyglutamine-binding protein 1 (PQBP1) gene. Previous studies show that PQBP1 binds to spliceosomal protein TXNL4A through its C-terminal domain, but the biological function of this interaction remains unclear. In this study, we found that TXNL4A was properly imported into the nucleus in the presence of wild-type PQBP1, but it was largely retained in the cytoplasm if its interaction with PQBP1 was disrupted by the mutations in PQBP1 C-terminal domain. PQBP1 is imported by karyopherin β2 through its PY-NLS. When we mutated its PY-NLS, PQBP1 was mislocalized to the cytoplasm as well as TXNL4A. Our data clearly demonstrate that the interaction between PQBP1 C-terminal domain and TXNL4A is critical for TXNL4A nuclear import. Mutations in PQBP1 C-terminal domain impair the import of splicing factor and affect mRNA splicing, which sheds light on the pathogenesis of Renpenning syndrome.

P2153/B400

The Novel Nuclear Targeting and BFRF1-interacting Domains of BFLF2 Are Essential for Efficient Ebv Virion Release.

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Epstein-Barr virus (EBV) genomic DNA is replicated and packaged into procapsids in the nucleus to form nucleocapsids, which are then transported into the cytoplasm for tegumentation and final maturation. The process is facilitated by the coordination of the viral nuclear egress complex (NEC), which consists of BFLF2 and BFRF1. By expression alone, BFLF2 is distributed mainly in the nucleus. However, it co-localizes with BFRF1 at the nuclear rim and in cytoplasmic nuclear envelope-derived vesicles in co-expression cells, suggesting temporal control of the interaction between BFLF2 and BFRF1 are critical for their proper function. The N-terminal sequence of BFLF2 is less conserved to that of alpha- and beta herpesvirus homologs. Here we found BFLF2 a.a. 2-102 is required for both nuclear targeting and its

interaction with BFRF1. Co-immunoprecipitation and confocal analysis indicated that a.a 82-106 of BFLF2 are important for its interaction with BFRF1. Three crucial amino acids (R47, K50, R52) and several non-continuous arginine and histidine residues within a.a. 59-80 function together as a non-canonical NLS, which can be transferred onto YFP-LacZ for nuclear targeting, in an importin β-dependent manner. Virion secretion is defective in 293 cells harboring a BFLF2 knockout EBV bacmid upon lytic induction, and is restored by trans-complementation of wild type BFLF2, but not NLS or BFRF1-interacting defective mutants. In addition, multiple domains of BFRF1 were found to bind BFLF2, suggesting multiple contact regions within BFRF1 and BFLF2 are required for proper nuclear egress of EBV nucleocapsids.

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Endosomes, Lysosomes, and Lysosome-Related Organelles 2

P2154/B402

Retromer Retrieves the Wilson Disease Protein, Atp7B from Lysosomes in a Copper-dependent Mode.

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The Wilson Disease protein, ATP7B utilizes lysosomal exocytosis to export copper out of liver. We investigated, what was the fate of ATP7B, post-copper export. At high copper ATP7B localizes to lysosomes and Rab7 endosome and upon copper chelation, returns to Trans Golgi Network. This retrograde trafficking of ATP7B is mediated by retromer complex. ATP7B co-localizes with Vps35 and Vps26, the core members of retromer. Knocking down of VPS35 or overexpressing the non-functional VPS35-R107A, did not alter TGN exit or vesicularization of ATP7B in response to copper; rather upon subsequent copper depletion ATP7B failed to relocalize to the TGN. Overexpressing the wtVPS35 rescued this non-recycling phenotype. On testing a series of sorting-motif mutants on ATP7B, we found that N-terminal motif NXXY mutant phenocopies retrograde trafficking behavior of ATP7B in VPS35 KD condition. Using Structured Illumination microscopy we demonstrate that VPS35 and ATP7B are juxtaposed on the same lysosomal compartment and possibly their interaction is indirect or transient. We demonstrate that retromer complex regulates lysosome to TGN trafficking of a non-resident lysosomal cargo, viz., ATP7B and that it is dependent upon copper conditions in the cell.

P2155/B403

Pleiotropic Role of Drosophila Ph*osphoribosyl Pyrophosphate Synthetase*In Autophagy and Lysosome Homeostasis.

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Phosphoribosyl pyrophosphate synthetase (PRPS) is a rate-limiting enzyme that plays a crucial role in nucleotide metabolism. The functional importance of PRPS is illustrated by the fact that human *PRPS1* is mutated in neurological disorders such as Arts syndrome, Charcot-Marie-Tooth disease, and nonsyndromic sensorineural deafness. However, it is currently unclear how PRPS deregulation contributes to neuropathogenesis and, as a consequence, no treatment option is available for affected patients. We identified the Drosophila ortholog of *PRPS* (*dPRPS*) as a direct target of RB/E2F in Drosophila, a vital cell cycle regulator, and engineered *dPRPS* alleles carrying patient-derived mutations. Two independent fly models of Arts syndrome-derived mutations established in this study, $dPRPS^{Q165P}$

and *dPRPS*^{R228W}, demonstrate shortened lifespan and locomotive defects, common phenotypes associated with neurodegeneration. While the flies develop normally, they are highly sensitive to nutrient deprivation caused by a failure to mobilize lipid reserves. We also show that *dPRPS* mutant flies have profound defects in macroautophagic induction and lysosome homeostasis, explaining why they fail to properly mobilize lipids upon starvation. Significantly, the nervous system of *dPRPS* mutant flies is affected by these defects and accumulate aberrant lipid droplets and protein aggregates in the brain. Interestingly, S-adenosylmethionine (SAM) supplementation, which is given to patients with Arts syndrome hoping to restore PRPS dysfunction, partially suppresses starvation sensitivity and improves lysosome dysfunction in *dPRPS* mutant flies. This suggests that the *dPRPS* mutants described in this study may be used to identify future therapies for *PRPS1*-associated disorders. Overall, we uncovered an unexpected link between nucleotide metabolism and autophagy/lysosome function and established a *Drosophila* model of PRPS-associated neurological disorder, providing a possible mechanism by which PRPS-dysfunction contributes to PRPS-associated neuropathology.

P2156/B404

HHV-7 U21 Exploits Golgi Quality Control Carriers to Reroute Class I MHC Molecules to Lysosomes.

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The human herpesvirus-7 (HHV-7) U21 glycoprotein binds to class I MHC molecules in the ER and reroutes them to lysosomes. How this single viral glycoprotein efficiently redirects the U21/class I MHC complex to the lysosomal compartment is poorly understood. To investigate the trafficking of HHV-7 U21, we followed synchronous release of U21 from the ER as it traffics through the secretory system, using live-imaging confocal and superresolution microscopy. Sorting of integral membrane proteins from the TGN has been shown to occur through tubular carriers that emanate from the TGN or through vesicular carriers that recruit GGA, clathrin adaptors, and clathrin (Chen et al., 2017). Here we present evidence for the existence of a third type of Golgi-derived carrier that is vesicular, yet clathrin-independent. This U21-containing carrier also carries a Golgi membrane protein engineered to form inducible oligomers (Galactosyl Transferase fused to FK506 dimerization domains). We propose that U21 employs the novel mechanism of forming large oligomeric complexes with class I MHC molecules that result in sorting of the oligomeric U21/class I MHC complexes to Golgi-derived quality control (QC) carriers destined for lysosomes.

P2157/B405

Actin-mediated Endosomal Recycling Is Regulated by Endosomal Cargo Load.

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Transmembrane proteins in the sorting endosome are either recycled to their point of origin or destined for lysosomal degradation. Lysosomal sorting is mediated by the interaction of ubiquitylated transmembrane proteins with the ESCRT machinery. We uncover an alternative role for the ESCRT-0 component HRS in promoting the constitutive recycling of transmembrane proteins. We find that endosomal localisation of the actin-nucleating factor WASH requires HRS, which occupies adjacent endosomal subdomains at steady state. Endosomal localisation of HRS-WASH is responsive to cargo load. Inhibition of receptor recycling leads to an accumulation of WASH on endosomal membranes at steady state. Whereas acute stimulation of cells with EGF lead to a reduction of endosomes associated

with WASH compared to HRS levels. Depletion of HRS results in defective constitutive recycling of epidermal growth factor receptor family (EGFR; ERBB2/Her2) and the matrix metalloproteinase MT1-MMP, leading to their accumulation in internal compartments. We show that direct interactions of receptors with endosomal actin are required for efficient recycling and use a model system of chimeric transferrin receptor trafficking to show that an actin-binding motif can counteract an ubiquitin signal for lysosomal sorting. In order to address how actin sorts cargo on the membrane we took advantage of a previously described actin binding domain in EGFR which is conserved in the ERBB family of proteins. Mutation of this site from EGFR and ERBB2 reduce egress rates of the receptors from the endosome indicating that direct actin binding to receptors facilitates their recycling. ERBB2 positive breast cancer invasion and metastasis is dependent on actin mediated recycling and patients show increased levels of WASH expression. WASH expression is increased in a model of acquired resistance against Trastuzumab based ERBB2 positive breast cancer therapy and inhibition of WASH function overcomes resistance. These findings point towards WASH complex regulation being directly linked to changes in cargo flux.

P2158/B406

pHLARE: a Genetically Encoded Ratiometric Lysosome pH Biosensor.

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Many lysosome functions are determined by a lumenal pH of ~5.0, including the activity of resident acid-activated hydrolases. Lysosome pH (pHlys) is increased in neurodegenerative disorders and predicted to be decreased in cancers, making it a potential target for therapeutics to limit the progression of these diseases. Accurately measuring pHlys, however, is limited by currently used dyes that accumulate in multiple intracellular compartments and cannot be propagated in cells. To resolve this limitation, we developed a genetically encoded ratiometric pHlys biosensor, pHLARE (pH Lysosomal Activity REporter), with the lysosome transmembrane protein LAMP1 tagged at the lumenal amino-terminus with superfolder GFP and the cytosolic carboxyl-terminus with mCherry. mCherry, with a pKa of 4.5 is insensitive to changes in the cytosolic pH range and allows ratiometric analysis normalized for abundance of the biosensor. We confirmed that pHLARE localizes predominantly in lysosomes, has a dynamic range of pH 4.0 to 6.5, and can be stably expressed in cells for propagation with cell division and for long-term time-lapse imaging. We developed a semi-automated image analysis algorithm to calibrate pHLARE fluorescence ratios, segment lysosomes, and determine the absolute pHlys of approximately a hundred lysosomes per cell. Using this pHLARE analysis, we show that inhibiting mTORC activity significantly decreases pHlys in epithelial cells from 5.22 ± 0.10 in controls to 4.63 ± 0.09. With pHLARE we also find that pHlys is significantly lower in triple negative MDA-MB-157 breast cancer cells (4.56 ± 0.11) and in PANC-1 pancreatic cancer cells (4.39 ± 0.19) compared with tissue-matched untransformed cells (5.36 ± 0.09 and 5.02 ± 0.18, respectively), and expression of oncogenic H-Ras-V12 decreases pHlys to 4.67 ± 0.07. Despite not being previously validated, decreased pHlys in cancers is consistent with increased activity of acid-regulated lumenal hydrolyases for metastasis and to increase biomass to fuel rapid proliferation. pHLARE is a new tool to accurately measure pHlys and for improved understanding of lysosome dynamics that could be a promising therapeutic target.

P2159/B407

Guanine Exchange Factor MADD Regulates Von Willebrand Factor Trafficking in Endothelial Cells Via Activation of Weibel-Palade Body-Associated Rab GTPases.

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Von Willebrand factor (VWF) is a secreted, multimeric protein that plays a crucial role in hemostasis. Endothelial cells secrete VWF via distinct pathways. Exocytotic vesicles containing small VWF multimers are continuously targeted primarily to the basolateral surface via the constitutive pathway (1), VWF of higher multimeric state is first stored in specialized organelles called Weibel-Palade bodies (WPBs) that steadily release their content through the basal secretion pathway (2) into the vascular lumen to maintain circulating VWF plasma levels. Upon vascular injury rapid stimulus-induced exocytosis of WPBs occurs via the regulated secretion pathway (3) to enable VWF string formation and subsequent platelet activation at the damaged site. Polarized secretion is crucial for the function of VWF at basolateral and apical surfaces, however, the regulation mechanism is unknown. Rab GTPases (Rabs) are a large family of proteins involved in vesicle trafficking. Several Rabs have been implicated in WPB trafficking, including the Rab3 isoforms Rab3B and Rab3D, as well as closely related Rab27A. Whether these WPB-Rabs are redundant or exhibit unique functions is still unknown. Rabs require a guanine nucleotide exchange factor (GEF) for their activation and recruitment to specific membranes. Previously, the Rab-GEF MAP-kinase activating death domain (MADD, also referred to as DENN or Rab3GEP) has been identified as a GEF for Rab27A and Rab3 isoforms in other cell types. Our research aims at elucidating if and how MADD is involved in regulating VWF secretion via these different secretory pathways. To investigate their role in WPB trafficking and secretion, Rab27A, Rab3B, Rab3D, and MADD were each targeted by shRNAs in human umbilical vein endothelial cells (HUVECs). Stable knockdown HUVECs were grown on Transwell membranes and unstimulated (basal) and histamine-evoked (regulated) VWF secretion was measured by ELISA. Unstimulated VWF secretion at the apical surface was decreased upon Rab27A, Rab3B, Rab3D, and MADD knockdown, but release on the basolateral side remained unchanged. Histamine-evoked VWF secretion showed a similar trend. Intracellular VWF content was reduced in Rab3D and MADD depleted cells, and immunofluorescent VWF staining revealed less WPBs. MADD silencing resulted in similar loss of peripheral localization of WPBs as observed in shRab27A treated cells. Based on our findings, we suggest a model in which MADD regulates apical VWF trafficking, through the activation and recruitment of Rab3D and Rab27A to WPBs.

P2160/B408

PQLC2 Signals Lysosomal Cationic Amino Acid Abundance to the C9orf72 Complex.

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The C9orf72 protein is required for normal lysosome function. In support of such functions, C9orf72 forms a heterotrimeric complex with SMCR8 and WDR41 that is recruited to lysosomes when amino acids are scarce. These properties raise questions about the identity of the lysosomal binding partner of the C9orf72 complex and the amino acid sensing mechanism that regulates C9orf72 complex abundance on lysosomes. We now demonstrate that an interaction with the lysosomal cationic amino acid transporter PQLC2 mediates C9orf72 complex recruitment to lysosomes. This is achieved through an interaction between PQLC2 and WDR41. The interaction between PQLC2 and the C9orf72 complex is

negatively regulated by arginine, lysine and histidine, amino acids that PQLC2 transports across the membrane of lysosomes. These results define a new role for PQLC2 in the regulated recruitment of the C9orf72 complex to lysosomes and reveal a novel mechanism that allows cells to sense and respond to changes in the availability of cationic amino acids within lysosomes.

P2161/B409

Modulation of Endo-lysosomal System in Mtb Infected Macrophages Mediated by Both Host Heterogeneity and Mycobacterial Lipids.

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Mycobacterium tuberculosis (Mtb) actively avoids fusion with late endosomes and lysosomes by inhibiting phagosomal maturation. While the mechanisms of phagosome maturation arrest at the level of mycobacterial phagosome are understood to some extent, the global impact of Mtb residence in arrested phagosome on the host cellular processes has not been systematically studied. In this study, we systematically explored the effect of Mtb infection on the host endo-lysosomal system. Our results show higher levels of different endosomal populations, endocytic uptake and lysosomes in Mtb infected cells compared to bystander-uninfected cells. Furthermore, comparison of endocytic profiles with other phagocytic cargos reveal that the observed alterations are not limited to Mtb but constitute a host-mediated effect to phagocytosis. Further dissection of the mechanisms involved identifies the endocytic heterogeneity in the host as a key determinant of the Mtb infection in macrophages. Unlike endosomes, lysosomal alterations are distinctly mediated by Mtb surface lipids in an infected cell. Mtb ΔWhiB3 mutant lacking the lipids elicits weaker lysosomal response suggesting the role of Mtb lipids in inducing lysosomal biogenesis. We find some of these lipids alter the lysosomal state of macrophages by acting upon mTORC1-TFEB axis. Further, abatement of the lysosomal system using pharmacological tools leads to enhanced intracellular Mtb survival, showing that the altered lysosomal state is a crucial determinant of the intracellular Mtb survival. Together, our study demonstrates that the host endocytic heterogeneity determines Mtb phagocytosis and further lysosomal rewiring mediated by Mtb lipids regulates homeostasis of an infected macrophage.

P2162/B410

Irs4 Mediates Egf-dependent Upregulation of Endocytosis through the Recruitment of Ras-pi3k Complex to the Endosome.

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Molecular dynamics, including changes in the subcellular localization of proteins, play an important role in determining the specificity and strength of intracellular signaling. We have previously reported that the small GTPase Ras, in complex with phosphoinositide 3-kinase (PI3K), translocates to the endosome upon epidermal growth factor (EGF) stimulation, and participates in the regulation of endocytosis and endosomal maturation (Tsutsumi *et al*., ***Cell. Signal.***21: 1672, 2009; Fujioka *et al*., ***PLoS ONE***6: e16324,

2011). More recently, we identified a peptide sequence within the Ras-binding domain of PI3K that dictates the endosomal localization of the Ras-PI3K complex and subsequent regulation of endocytosis (Fujioka *et al*., ***Cell Struct. Funct.***44: 61, 2019). This peptide was named RAPEL after Ras-PI3K endosomal localization. The detailed molecular mechanism through which the complex is recruited to the endosome has yet to be determined, however. Here we report that insulin receptor substrate 4 (IRS4), which had been identified as a RAPEL-binding protein through mass spectrum analysis, translocates to the endosome by EGF stimulation, and recruits the Ras-PI3K complex to this organelle. The knockdown of IRS4 resulted in the inability of the Ras-PI3K complex to localize to the endosome and inhibition of clathrin-independent endocytosis. IRS4 is likely to be recruited to the endosome through two distinct mechanisms: i.e. Through the pleckstrin homology (PH) domain in a quiescent state and through tyrosine phosphorylation by Src in the presence of EGF. Our results might provide novel mechanistic insights into how endocytosis is controlled by the spatiotemporal regulation of signaling molecules.

P2163/B411

Type II Phosphatidylinositol 4-Kinases Modulate BLOC-1-Dependent Tubular Transport Carrier Formation from Endosomes during Melanosome Biogenesis.

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Melanosomes are pigment cell-specific lysosome-related organelles (LROs) in which melanin pigments are synthesized and stored. Melanosome maturation from non-pigmented precursors requires biosynthetic delivery of melanogenic enzymes and transporters from early endosomes (EEs) by two pathways. One essential pathway requires the multisubunit complex, BLOC-1, on EEs to generate tubular transport intermediates through which cargoes such as TYRP1 are delivered to maturing melanosomes. The molecular mechanism by which BLOC-1 facilitates tubule formation is incompletely understood, but recent studies suggest that phosphatidylinositol-4-phosphate (PtdIns4P) and the type II PtdIns 4-kinases (PI4KIIα and PI4KIIβ) might play a role. Moreover, in other cell types PI4KIIα and PI4KIIβ interact respectively with AP-3 and AP-1, which also play roles in BLOC-1-dependent cargo delivery. We hypothesized that PI4KIIα and/or PI4KIIβ regulate EE tubule formation by BLOC-1. By live-cell imaging, both PI4KIIα and PI4KIIβ are detected on melanosome-bound EE tubules. Transient depletion of either PI4KIIα or PI4KIIβ by shRNA impaired melanosome localization of TYRP1 by immunofluorescence microscopy (IFM), as also observed in BLOC-1-depleted cells; however, unlike in BLOC-1-depleted cells, the mislocalized TYRP1 in PI4KII-depleted cells accumulated in late endosomes/ lysosomes and not extensively in EEs. Moreover, depletion of either PI4KIIα or PI4KIIβ in wild-type melanocytes did not affect the association of BLOC-1 with membranes by subcellular fractionation, and in BLOC-1-knockout melanocytes did not prevent TYRP1 accumulation in EEs by IFM. Live imaging showed that deficiency of PI4KIIα, but not of PI4KIIβ, impairs EE tubule length without blocking tubule formation. These results

indicate that PI4KIIα and PI4KIIβ are each required downstream of BLOC-1 for sequential steps in BLOC-1-dependent cargo delivery to melanosomes. They further suggest that PI4KIIα and PI4KIIβ are not required for BLOC-1-mediated tubule initiation but may be required for tubule elongation or stabilization (PI4KIIα) and/or targeting to melanosomes (PI4KIIβ). Our study demonstrates an important role of type II PtdIns 4-kinases in BLOC-1-dependent tubular cargo transport during melanosome biogenesis and extends the cohort of effectors required for LRO biogenesis.

P2164/B412

Ampk Contributes to Autophagosome Maturation and Lysosomal Fusion.

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AMP-activated protein kinase (AMPK) regulates autophagy initiation when intracellular ATP level decreases. However, the role of AMPK during autophagosome maturation is not fully understood. Here, we report that AMPK contributes to efficient autophagosome maturation and lysosomal fusion. Using CRISPR-Cas9 gene editing, we generated AMPK α1 knockout HEK293T cell lines, in which starvation-induced autophagy is impaired. Compound C, an AMPK-independent autophagy inducer, and trehalose, an mTOR-independent autophagy inducer were used to examine the role of AMPK in autophagosome maturation and lysosomal fusion. While the treatment of control cells with either compound C or trehalose induces activation of autophagosomes as well as autolysosomes, the treatment of AMPK α1 knockout cells with compound C or trehalose induces mainly activation of autophagosomes, but not autolysosomes. We demonstrate that this effect is due to interference with the fusion of autophagosomes with lysosomes in AMPK α1 knockout cells. The transient expression of AMPK α1 can rescue autophagosome maturation. These results indicate that AMPK α1 is required for efficient autophagosome maturation and lysosomal fusion.

P2165/B413

Presenilin 1 Functions in Endosomal Recycling to Maintain Lysosomal Clearance.

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Autophagic and endosomal dysfunction observed at preclinical stages of Alzheimer's disease (AD) or in Presenilin (PSEN) 1 -/- mouse neurons are suggested to be related to the non-catalytic role of PSENs in lysosomal homeostasis. The precise molecular mechanism accounting for these alterations remains nonetheless debated, and several hypothesis have emerged over the years, most of them focusing on the alteration of lysosomal function. Previously reported lipidome and proteome analysis of the plasma membrane of PSEN deficient cells have revealed a selective depletion of (i) proteins involved in cell adhesion/migration, transport regulation, signaling and transporters, as well as (ii) a significant depletion of cholesterol. Accordingly endosomal cargoes depleted from the plasma membrane were shown to accumulate intracellularly, highlighting the existence of defects in specific endosomal transport routes to and/or from the cell surface. To re-investigate the role of PSEN1 in endosomal transport regulation, we generated independent clones of PSENdKO mouse embryonic fibroblasts and subsequently rescued those with hPSEN1 and the catalytic inactive mutant variant PSEN1 D257A/D385A, to ensure isogenic backgrounds. These PSENdKO cells present with enlarged late endosomes/lysosomes that (i) display an increased co-localization with early endosomal markers and (ii) also accumulate cargo

such as CI-M6PR and VPS35, a key subunit of the retromer involved in cargo recognition and sorting. These accumulations were relieved upon re-expression of either WT or catalytically inactive PSEN1, indicating that a γ-secretase-independent role of PSEN1 is functionally connected to recycling. Interestingly, similar defects were observed when knocking down ARF6, a small GTPase involved in membrane traffic/clathrin-independent endocytosis and a key regulator of cholesterol trafficking. Conversely, overexpression of ARF6 in PSENdKO cells alleviated partially endolysosomal defects. All together, these results involve PSEN1 in the control of proper endosomal recycling, and this independently from its γ-secretase activity. Age-related alterations in the expression of PSEN1 may therefore potentially affect AD etiology independent from its catalytic function.

P2166/B414

Endolysosome Ferrous Iron Concentrations Are Quantitatively Diverse and Labile.

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Endosomes and lysosomes (endolysosomes) are acidic organelles that are important both physiologically and pathologically. Implicated in the physiological and pathophysiological processes are readily releasable stores of cations including ferrous iron (Fe^{2+}); an essential cofactor for various enzymes and the generation of reactive oxygen species. To determine the extent to which and mechanisms by which endolysosome Fe^{2+} affects cellular functions, it is important to first determine levels of endolysosome Fe^{2+}. Here, using U87MG astrocytoma cells and primary cultures of rat cerebral cortex neurons we found that FeRhoNox-1 was highly specific for Fe^{2+} and that FeRhoNox-1 positive stores were highly localized in endolysosomes. Control levels of endolysosome Fe^{2+} were 36.6 ± 13.6 µM; endolysosome Fe^{2+} levels increased to 75 ± 15.7 µM when cells were treated with ferric ammonium citrate and decreased to 0.08 ± 0.05 µM when cells were treated with the iron chelator deferoxamine. The Fe^{2+} content in endolysosomes was heterogeneously distributed; findings that suggest subpopulations of endolysosomes. Endolysosomes with higher levels of Fe^{2+} were localized near the nucleus while endolysosomes with lower levels of Fe^{2+} were localized near plasma membranes. Thus, FeRhoNox-1 can be used to label and measure levels of endolysosome Fe^{2+}. In doing so, this will aid in the determination of the importance of Fe^{2+} in subpopulations of endolysosomes, their locations in cells, and the effects of released endolysosome Fe^{2+} on downstream cellular events. (This work was supported by the following grants; MH100972, MH105329, MH119000, DA032444.)

P2167/B415

Structural Mechanism of Folliculin-mediated Regulation of the Rag Gtpase Activation Cycle.

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The decision to allocate resources toward cellular growth or quality control is critical for organismal survival. The mechanistic Target of Rapamycin Complex I (mTORC1) tunes the balance between cellular anabolism and catabolism, and is one of the most frequently mutated pathways in cancer. In response to nutrient stimulation, the Rag GTPases (Rags) recruit mTORC1 from the cytosol to the lysosomal surface, where its kinase function is activated. The Rags are obligate heterodimers (Rag A:RagC), whose nucleotide binding states respond to cellular nutrient status and determine their ability to bind to mTORC1. A key but poorly understood event in mTORC1 activation is conversion of RagC from the GTP-

to the GDP-bound form. A complex of Folliculin (FLCN) and FLCN-interacting protein (FNIP) was proposed to promote mTORC1 activation by functioning as a RagC -specific GTPase Activating Protein (GAP), but how RagC is activated at the right time by FLCN:FNIP, and how it remains in check until needed, is one of the major unanswered questions in the mTORC1 regulation field. To determine the regulatory functions of FLCN:FNIP, we reconstituted a 'lysosomal FLCN complex' (LFC) containing FLCN:FNIP, the starved-state $RagA^{GDP}$:$RagC^{GTP}$ GTPases, and the lysosomal anchor Ragulator and determined its cryo-EM structure to 3.6Å. Surprisingly, this complex inhibits the RagC-GAP activity of FLCN by displacing the catalytic Arg finger of FLCN away from the RagC nucleotide pocket. Conversely, within the LFC, FLCN:FNIP protects starved-state $RagA^{GDP}$, providing a mechanism for stabilization of the inactive state Rag complex. Together, these data identify the LFC as a novel activation checkpoint for mTORC1, whereby FLCN:FNIP may help maintain the Rags in an inactive state under low nutrients, and yet is necessary for conversion to the active state when nutrient levels rise.

P2168/B416

Repair of Plasma Membrane Wounds Caused by Bcr-antigen Interaction Is Mediated by Lysosomal Exocytosis and Promotes Antigen Uptake.

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B lymphocytes survey antigen through the B cell receptor (BCR), which initiates the activation of B cells to mount antibody responses against infectious agents. Polarization of the BCR and lysosomes towards the antigen interaction site are important steps that lead to antigen acquisition and presentation to T cells. Lysosomal enzymes were proposed to participate in extraction of surface-immobilized antigen by B cells (1), but the trigger for lysosomal enzyme secretion at the B cell immunological synapse remained unknown. Plasma membrane (PM) wounding triggers lysosomal exocytosis, a process required for PM repair in several cell types (2). We previously showed the importance of lysosomal exocytosis in the repair of B cell PM wounds caused by pore-forming toxins (3). This study addresses whether PM wounding occurs when B cells engage antigen, and whether PM wounding and repair affect antigen acquisition. We found that pseudo and bona fide antigen but not transferrin, when immobilized to beads or planar lipid bilayers, wound the B cell PM in an antigen dose-dependent manner. The actin motor non-muscle myosin II (NMII) and the BCR polarize towards antigen interaction sites before PM wounding, and NMII inhibition reduces the number of wounded cells. The lysosomal protein Limp2 is exposed at sites of antigen engagement, and inhibition of lysosomal exocytosis or the lysosomal enzyme acid sphingomyelinase increase the number of wounded cells. Thus, antigen-induced PM wounding is followed by lysosome-mediated PM repair. Antigen internalization occurs after polarization of lysosomes towards antigen-binding and PM wounding sites, and is decreased when antigen-induced PM wounding is reduced by the NMII inhibitor. These results suggest that forces generated by NMII within B cells following engagement of immobilized antigen by the BCR can wound the PM, and that lysosome-mediated wound repair can facilitate internalization of antigen from presenting surfaces. This work is supported by the NIH grant GM064625 to NWA and WS.1. Yuseff, M-I, Reversat, A, Lankar, D, Diaz, J, Fanget, I, Pierobon, P, Randrian, V, Larochette, N, Vascotto, F, Desdouets C, Jauffred B, Bellaiche, Y, Gasman, S, Darchen, F, Desnos, C and Lennon-Duménil, A-M. *Immunity* 35: 361-374, 2011. 2. Reddy, A, Caler, E V and an drews, N W. *Cell* 106: 157-169, 2001. 3. Miller, H, Castro-Gomes, T, Corrotte, M, Tam, C, Maugel, TK, an drews, NW and Song, W. *J. Cell Biol.* 211: 1193-1205, 2015.

P2169/B417

Regulation of Cholesterol-dependent Mtorc1 Signaling by Inter-organelle Contacts.

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Organelle communication is key to cellular homeostasis, and its failure is an emerging driver of neurodegenerative and metabolic disease. Cholesterol, an essential building block for membranes and precursor for growth hormones, is exchanged by specialized sterol carriers located at physical contacts between the endoplasmic reticulum (ER) and other organelles. Recently, cholesterol was identified as an essential input that activates the master growth regulator, mTORC1 kinase, by promoting its recruitment to the lysosomal limiting membrane via the Rag guanosine triphosphatases (GTPases). Cholesterol triggers major programs downstream of mTORC1, including upregulation of anabolism and suppression of autophagy, but the mechanisms that regulate lysosomal cholesterol content to enable mTORC1 signaling are unclear. More generally, whether and how inter-organelle contacts govern cell-wide programs for growth and quality control is not understood. Here we show that Oxysterol Binding Protein (OSBP) and its anchors at the ER, VAPA/B, deliver cholesterol across ER-lysosome contacts to activate mTORC1. In cells depleted for OSBP, but not other VAP-interacting cholesterol carriers, mTORC1 recruitment by the Rag GTPases is inhibited due to impaired cholesterol transport to lysosomes. Moreover, in cells lacking the lysosomal cholesterol exporter, Niemann-Pick C1 (NPC1), unopposed ER-to-lysosome transport by OSBP drives aberrant buildup of cholesterol on the lysosomal limiting membrane. Consequently, genetic and small molecule-mediated inhibition of OSBP selectively reverses cholesterol accumulation on the limiting membrane but not in the lumen of NPC1-null lysosomes, suppresses aberrant mTORC1 signaling and restores defective autophagy, a major driver in the pathogenesis of Niemann-Pick type C. Thus, ER-lysosome contacts emerge as signaling hubs that enable cholesterol sensing by mTORC1, and their manipulation via OSBP inhibitors could be beneficial in Niemann-Pick type C and mTORC1-driven diseases.

P2170/B418

A Forward Genetic Screen to Identify Novel Regulators of Vacuole Inheritance.

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Organelles such as the endoplasmic reticulum, mitochondria, and lysosomes are inherited during eukaryotic cell division. Little is known about the pathways that regulate organelle inheritance or how it is coordinated with the cell cycle. The baker's yeast, Saccharomyces cerevisiae, partitions its vacuole and transports a portion of it into the growing daughter cell during budding. The vacuole is similar to the mammalian lysosome and is responsible for critical metabolic processes such as macromolecule recycling, ion homeostasis, and cell cycle progression. These aspects make budding yeast an ideal organism for studying organelle inheritance. While some of the mechanisms that regulate vacuole inheritance have been uncovered, we hypothesize that there are many components of the pathway that remain to be discovered. To find novel regulators of vacuole inheritance, we are conducting a forward genetic screen to identify mutants in the pathway. We began by treating yeast cells with a red

fluorophore, FM4-64, which specifically labels the vacuole. This labelling allows us to visualize the yeast vacuole either by fluorescence microscopy or flow cytometry. Mutants in vacuole inheritance that are unable to transport the vacuole during budding accumulate vacuoles in the mother cell and lack vacuoles in daughter cells. After labelling the vacuoles, we compared the flow cytometry profiles of known vacuole inheritance mutants with wild-type cells. We assessed both cell size and fluorescence and determined properties that distinguished between mutant and wild-type yeast. We then treated wild-type yeast with the chemical mutagen, ethyl methanesulfonate, and used a fluorescence activated cell sorter along with our predetermined paramaters to enrich for vacuole inheritance mutants. Potential hits were isolated and visualized under the microscope to determine if they have a vacuole inheritance defect. This technique enabled us to identify approximately one mutant colony per fifty colonies screened. We have currently found several mutants that cannot properly inherit the vacuole. After screening we will identify the causative mutations using pooled linkage analysis and whole genome sequencing. Further experiments will include conducting biochemical and genetic assays to determine binding partners of these proteins as well as their catalytic activity. Overall, these studies will identify novel regulators of vacuole inheritance.

P2171/B419

The Lipid Kinase, Vps34, Is a Driver of Selected Membrane Trafficking Pathways.

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Phosphoinositide lipids are low-abundance signaling molecules that control signal transduction pathways and are necessary for cellular homeostasis. The phosphoinositide lipid, phosphatidylinositol 3-phosphate (PI3P), recruits downstream effector proteins to control a wide-range of membrane trafficking events. In the budding yeast, *Saccharomyces cerevisiae*, Vps34, a phosphatidylinositol 3-kinase, is the sole source of PI3P. PI3P is required for several cellular processes including synthesis of $PI3,5P_2$, retromer function, autophagy, and formation of multivesicular bodies via the ESCRT machinery. Moreover, PI3P levels are acutely elevated in response to some stimuli, which suggests that dynamic changes in PI3P drive some downstream pathways. This possibility would not be revealed in most studies, which utilize knock-down or knock-out approaches. Here we seek to extend current knowledge by determining the consequences of increasing PI3P levels. We designed a hyperactive mutant based on a high-resolution structure (Rostislavleva et al., Science 2015) combined with a study that suggested that activation of Vps34 occurs in part via dissociation of the Vps34 kinase domain from its scaffold protein, Vps15 (Stjepanovic et al., Mol. Cell 2017). In addition, we identified Vps34 mutants that increase PI3P levels by screening for mutants that suppress an impaired growth phenotype of a catalytically damaged Vps34 allele. These hyperactive mutants increase cellular PI3P as well as $PI3,5P_2$ levels both at basal conditions and during hyperosmotic shock. Study of these hyperactive mutants has revealed that elevation of PI3P selectively drives certain trafficking pathways while not affecting, or even inhibiting others. Hyperactive Vps34 alleles were found to enhance Snx4-dependent retrograde traffic of Atg27 from the vacuole membrane. In contrast, ESCRT- and membrane fusion-dependent degradation of the plasma membrane methionine transporter, Mup1, following addition of methionine, is unchanged by elevated PI3P. Finally, our data suggests that while autophagy induction is unchanged by excess PI3P, increasing PI3P levels correlates with increasing defects in the degradation of autophagy cargoes. This phenotype may be due to defects in maturation of autophagosomes, their fusion with the vacuole, and/or the proteolytic capacity of the vacuole. Taken together, our data suggests that changes in phosphoinositide lipid levels may provide a way for cells to acutely respond to some stimuli. More

broadly, the approach of using hyperactive mutants can be applied to other lipid kinases and may contribute insights into phosphoinositide lipid signaling.

P2172/B420

Programmable Signaling Endosome Nexus in Regulating Receptor Recycling and Cancer Cell Migration.

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Phenotypic plasticity of cancer is modulated by multiple mechanisms including altered receptor trafficking and signaling downstream of gain-of-function (GOF) mutant p53. Reciprocal crosstalk of endocytic trafficking and receptor signaling function as a flexible network to increase the complexity of signaling output to regulate tumor plasticity. We report that neuronal-enriched dynamin-1 isoform (Dyn1) is up-regulated at both the mRNA and protein levels in a manner dependent on expression of GOF mutant p53. Dyn1 is required for the recruitment and accumulation of the signaling scaffold, APPL1, to a spatially localized subpopulation of endosomes at the cell perimeter. The perimeter APPL1 endosomes modulate Akt signaling and activate Dyn1 to create a positive feedback loop required for rapid recycling of EGFR and β1 integrins, increased focal adhesion turnover, and cell migration. Thus, Dyn1- and Akt-dependent perimeter APPL1 endosomes function as a nexus that integrates signaling and receptor trafficking, which can be co-opted and amplified in mutant p53-driven cancer cells to increase migration and invasion. This work exemplifies the signaling endosomes are programmable in response to signaling to create positive and/or negative feedback loops to regulate cancer cell phenotypic plasticity. More work is needed to further disclose the role of programmable signaling endosomes in modulating cancer progression.

Post-Golgi Trafficking

P2173/B421

Dopey1-Mon2 Complex Binds to Dual-lipids and Recruits Kinesin-1 for Centrifugally Biased Bidirectional Trafficking Around the Golgi.

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In the secretory and endocytic pathway, the centripetally positioned Golgi complex exchanges membrane carriers with centrifugally or peripherally positioned organelles such as the ER, endolysosome and plasma membrane (PM). While it is known that microtubule motors can drive organelle positioning and membrane trafficking along microtubule tracks, it is currently unclear how they work at the molecular and cellular level in *vivo*. We found that Dopey1 and Mon2, two poorly characterized Golgi peripheral membrane proteins, can assemble into a novel complex. The Dopey1-Mon2 complex is a heterotetramer comprising a Mon2 homodimer interacting with two copies of Dopey1. Within the complex, three novel domains were identified that can directly interact with KLC2 subunit of kineisn-1 holoenzyme, phosphatidylinositol-4-phosphate (PI4P) and phosphatidic acid (PA). The membrane association of the complex requires both PI4P and PA. We uncovered that the complex functions as a kinesin-1 adaptor by linking PI4P-PA-positive organelles, including the Golgi, ER exit site and endolysosome, and carriers budded from them. Through extensive quantitative imaging, we systematically investigated the complex's function in organelle positioning and membrane trafficking to and from the Golgi. We discovered that it is essential for the peripheral positioning of the ER exit site and endolysosome and the expansion of the Golgi; it promotes the centrifugally directed trafficking

pathways, including the Golgi-to-PM and Golgi-to-ER, and inhibits the centripetally directed ones, including ER-to-Golgi and PM-to-Golgi. In conclusion, Dopey1-Mon2 complex is a dual-lipid-regulated cargo-adaptor that recruits kinesin-1 motor for centrifugally biased bidirectional transport around the Golgi. The identification of Dopey1-Mon2 complex therefore provides an important missing link to coordinate the budding of a membrane carrier and subsequent bidirectional transport along the microtubule network.

P2174/B422

Lack of Rho Activity Contributes to Transcytotic Apical Protein Targeting in Hepatocytes.

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Protein targeting within cells is crucial for maintaining cell function. In columnar epithelial cells, such as those of the kidney, targeting apical and basolateral proteins to different plasma membrane domains from the *trans*-Golgi network (TGN), requires their segregation into distinct transport carriers. Hepatocytes target apical proteins predominantly by means of a mechanism known as transcytosis in which TGN-derived cargo is targeted first to the basolateral sinuisoidal domain before its arrival at the apical canalicular domain. The molecular mechanisms underlying transcytotic apical targeting in hepatocytes are poorly understood. Specifically it is unknown whether apical and basolateral proteins are segregated during TGN to surface targeting. Here we examined the exit and trafficking of apical (human dipeptidyl peptidase IV) and basolateral (vesicular stomatitis virus glycoprotein) cargo in live hepatocytic epithelial cells by dual-colour confocal and TIRF microscopy. We observed a predominant simultaneous exit of apical and basolateral proteins from the TGN into common tubular carriers that eventually connect and fuse with plasma membrane in hepatocyte-derived WIF-B9 cells and primary cultures of hepatocytes. Rho activation decreases the formation of tubular TGN-derived carriers and promotes the segregation of apical from basolateral proteins into separate vesicles. These findings suggest the lack of a Rho-dependent scission mechanism at TGN-derived tubules in hepatocytic cells, a phenotype that potentially contributes to the hepatocyte transcellular transport function.

P2175/B423

Ric1 Regulates Trans-golgi Trafficking of Procollagen and Is Linked to a Complex Human Syndrome.

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Procollagen presents a unique challenge for the secretory pathway because of its bulky rod shape and large secretory volumes during organ morphogenesis. Components of the early secretory pathway machinery transporting extracellular matrix molecules (ECM) were identified, but the distal, post-Golgi components are sparsely known. Using a zebrafish phenotype-driven forward genetic screen and positional cloning of the *round*mutations, we discovered that the *ric1*gene is essential for normal skeletal biology. We show in vivo and in cell culture that activation of Rab6a GTPase by the Ric1-Rgp1 GEF complex is required for procollagen secretion through the Trans-Golgi-Network (TGN) en route to extracellular space. The requirement for the GEF can by bypassed by the constitutively active Rab6a and leads to cell autonomous release of the backlogged collagen. Zebrafish*round*mutations cause chondrocyte dysfunction, musculoskeletal and cranio-dental malformations. Quantitative genetics approaches using gene-based PheWAS by PrediXcan in the BioVU biobank revealed highly significant

associations with skeletal and dental defects in patients with reduced genetically predicted expression of the *RIC1*gene, thereby corroborating the zebrafish phenotypic findings. Furthermore, individuals homozygous-by-descent for a rare variant in *RIC1*were discovered to have many signs of the BioVU associated phenome including cleft lip, cataract, tooth abnormality, intellectual disability, facial dysmorphism and ADHD. Zebrafish and patient skin fibroblasts accumulate intracellular procollagen in TGN, and the human *RIC1*variant fails to rescue the procollagen secretory defects in zebrafish *round*mutants, unlike wild type *RIC1,*establishing the pathogenicity of the variant and evolutionary conservation of this novel procollagen secretory pathway. Taken together, our data indicate that RIC1-RGP1 complex is essential for collagen secretion and normal human development

P2176/B424

Visualization and Molecular Dissection of Collagen IV Secretion in Mouse Epithelial Cells.

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Collagen IV is a major component of the basement membrane matrix that demarcates epithelial tissues. To facilitate the secretion of 400 nm-long collagen IV protomers, collagen IV mRNAs are known to be basally polarized in follicle epithelial cells of the Drosophila egg chamber. In contrast, we observed unexpected strong apical polarization of collagen IV mRNAs in the outer epithelial cells of mouse embryonic salivary glands using single molecule RNA fluorescence in situ hybridization (smFISH). Moreover, both the endoplasmic reticulum (ER) and the Golgi apparatus were also apically polarized in these cells, indicating the site of collagen IV production was opposite to its site of deposition. To visualize intracellular trafficking, we constructed lentivirus vectors expressing mNeonGreen-tagged collagen IV. The tagged collagen IV could incorporate into the basement membrane of lentivirus-transduced salivary glands, indicating it is at least partially functional. Separately, we used lentiviruses to establish a clonal mouse epithelial cell line stably expressing both mNeonGreen-tagged collagen IV and Cas9. These cells can secrete a dense layer of fluorescent matrix on the ventral (substrate) side. Inside these cells, we observed dynamic collagen IV vesicles with no obvious patterns of movement, presumably because the vesicles were at different stages of secretion. To elucidate the dynamics of secretion, we established a RUSH (retention using selective hooks) assay to synchronize collagen IV secretion by co-expressing an ER-resident streptavidin and a collagen IV subunit tagged with a streptavidin binding peptide (SBP) and mScarlet. We showed that biotin addition quickly released collagen IV from ER retention, and it first became enriched at the Golgi and then secreted from the cells to incorporate into the underlying matrix. Using CRISPR/Cas9, we showed that the amount of collagen IV secretion was reduced or eliminated when Serpinh1 (the collagen-specific chaperone HSP47), Mia3 (mammalian Tango1 homolog required for large COPII vesicle formation), Kif5b (a transport kinesin), or Rab6a were each knocked out. We further demonstrated that the collagen IV secretion eliminated in clonal Rab6a knockout cells could be rescued by expressing mScarlet-Rab6a. On the other hand, Rab10 knockout completely disrupted the polarized distribution of secreted collagen IV matrix without significantly affecting the amount of secretion. In summary, we have established a system for visualizing synchronized collagen IV secretion and demonstrated the importance of several molecular machineries for collagen IV secretion in mouse epithelial cells. We will further pursue the mechanisms underlying polarized collagen IV secretion and evaluate these findings in mouse salivary glands.

P2177/B425

A Complex of Conserved Proteins Regulates Ap-1 Functions in Yeast.

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The clathrin adaptor protein complex-1 (AP-1) plays a central role in membrane traffic at the *trans*-Golgi network (TGN). It is thought to mediate protein sorting by forming a physical link between clathrin and its membrane embedded cargo. The association of AP-1 with membranes is therefore critical for accurate proteins sorting. Several factors are known to contribute to AP-1 membrane association including phosphatidyl-inositides and Arf-GTPases. In addition, proteins of the conserved HEATR5 family play a conserved but poorly understood role in AP-1 membrane association. Although HEATR5 proteins were identified in AP-1 interactomes, HEATR5 proteins do not contain obvious AP-1 binding sequences, suggesting that their interactions with AP-1 are indirect. Indeed, the mammalian family member HEATR5b is known to interact with two proteins that themselves directly interact with AP-1. However, neither protein is conserved outside of animals suggesting that they may perform activities unique to animal cells. In order to better understand conserved HEATR5 functions, we used a combination of bioinformatics techniques and mass-spectrometry of purified complexes to identify binding partners for the yeast HEATR5 protein Laa1. Combined these approaches identified two conserved co-factors of Laa1 that are essential for the normal association of AP-1 with the TGN. We found that the co-factor Laa2, a likely homolog of mammalian Clba1, binds directly to AP-1. We mapped the interaction of Laa2 with AP-1 to a single canonical AP-1 binding motif in the N-terminus of Laa2. We found that this interaction is essential for the normal association of AP-1 with the TGN in yeast. This approach also identified a poorly characterized GTPase binding protein as a Laa1 binding co-factor. We found that this co-factor is required for Laa1 and AP-1 membrane association. This suggests that Laa1 may integrate signals from multiple GTPases at the TGN. Together these results indicate that Laa1 functions in a complex with other conserved proteins that works in parallel with phosphatidyl-inositides and Arf-GTPases to mediate the stable membrane association of AP-1.

P2178/B426

Distinct Roles for Secretory Pathway Acidification during Apical Membrane Biogenesis in Enterocytes.

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Epithelial cell physiology critically depends on the asymmetric distribution of channels, transporters and other membrane proteins. However, little is known of how proteins are targeted to the apical membrane in vivo. Here, we performed a visual forward genetic screen in zebrafish and identified recessive mutants defective in apical targeting of p75, an O-glycosylated membrane protein, in the intestine. One of these mutants, atp6ap1, revealed specific requirements for luminal acidification in apical, but not basolateral, membrane protein transport. Using a visual assay based on the classical low temperature block, we were able to monitor transport of newly synthesized membrane proteins from the trans-Golgi network to the plasma membrane in live zebrafish larvae. Combining this approach with genetic and pharmacologic perturbation of luminal pH, we show that vacuolar H+ ATPase (V-ATPase) activity regulates sorting of O-glycosylated proteins at the TGN as well as Rab8-dependent trafficking of apical membrane proteins. Thus, luminal acidification plays distinct and specific roles in apical membrane biogenesis.

P2179/B427

Unfolded Protein Response Regulates Arf-GAP Gcs1 Phosphorylation to Modulate Arl1 GTPase Activation.

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ADP-ribosylation factor (Arf) and Arf-like (Arl) small GTPases are known to play important roles in membrane trafficking. In *Saccharomyces cerevisiae*, the guanine nucleotide-binding cycle of Arl1 is converted by the guanine nucleotide exchange factor (GEF), Syt1, and the GTPase activating protein (GAP), Gcs1. The induction of ER stress affects many molecular pathways that cause the unfolded protein response (UPR). We previously demonstrated that UPR signaling regulated phosphorylation of Syt1, which is critical for Arl1 activation and recruitment of golgin protein Imh1 to the Golgi. However, the detailed mechanism of how Gcs1 is regulated to access the Golgi membrane and promote GTP-hydrolysis of Arl1 upon UPR induction remains unclear. Based on the prior large-scale screening, Gcs1 harbors numerous putative phosphorylation sites. Here, we showed that Gcs1 was phosphorylated upon treatment of UPR-inducer tunicamycin. By mass spectroscopy, we identified several phosphorylation sites on Gcs1 upon UPR activation. We observed that the phosphorylation decreased the association of Gcs1 to the Golgi membrane. The phosphorylated Gcs1 weakens its ability to access Arl1 that supports an increase of Arl1 and Imh1 puncta under UPR activation. Interestingly, the trigger of Gcs1 phosphorylation upon UPR activation is not dependent on a well-known Ire1-Hac1 signaling pathway, but rather a Slt2-related pathway. Furthermore, Slt2-deletion attenuates an increase of Arl1-mediated golgin Imh1 puncta upon UPR induction. Thus, we infer that the regulation of Gcs1 phosphorylation is critical for modulating the Arl1 function upon ER stress.

P2180/B428

Ags3 Transport E-cadherin to Memberane during Embryonic Development in Mice.

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Activator of G protein signaling 3 (AGS3) alters trans-golgi network structure and function by binding of its GoLoco motif (GPR domain) to Gαi and activating G protein signaling, and therefore regulates transport of proteins to plasma membrane. Compaction of early embryo is based on accumulation of E-cadherin at cell-contect membrane. However, whether and how AGS3 regulates polar transport of E-cadherin to plasma membrane are unknown. To make these clear, AGS3 of mouse early embryo was knocked out by Cas9-sgRNA systerm. In addition, trans-golgi network protein 46 (TGN46) and transmembrane P24 trafficking protein 7 (TMED7) were traced in embryos by fusion to fluorescent protein label. In results, embryos in control group successfully developed to blastocyst stage, E-cadherin was localizated at cell-contect membrane, and trans-golgi network and TMED7-positive vesicle gradually aggregated near to cell-contect membrane; however, most of *AGS3*-edited embryos in knockout group were arrested and fragmented since 4-cell stage with decreased accumulation of E-cadherin at the membrane, trans-golgi network and TMED7-positive vesicle were dispersive and could not be polarized to the membrane. In addition, decreased Gαi3 could rescued these *AGS3*-edited embryos in knockout group. These results indicate that AGS3 reinforces dynamic of trans-golgi network from 4-cell to MO stage by binding and activating Gαi3 at golgi complex. Activated Gαi3 disassociates from Gβγ and it

resulted in activation of G protein signaling at TGN and transport of TMED7-positive cargo containing E-cadherin to cell-contact membrane during mouse early embryo development.

P2181/B429

The Golgin Imh1 Mediates Recycling Transport of SNARE Upon ER Stress.

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Eukaryotic cells have developed conserved pathways to deal with the stress coming from accumulation of unfolded protein in ER. Induction of unfolded protein response (UPR) in yeast has been reported to enhance Arl1 GTPase activation, which promotes the recruitment of additional golgin Imh1 to the trans-Golgi network (TGN). Golgins play important roles in tethering membrane fusion and maintaining the structure of Golgi. However, it is unclear whether induction of additional golgin Imh1 structures associated with amplification of the TGN can alleviate the ER stress. Here, we report that Imh1 is required for transporting exocytic v-SNARE Snc1 from TGN to the plasma membrane upon induction of UPR. Snc1 is a v-SNARE which exocytoses to the plasma membrane and then recycles back to TGN through endosome. We first found that both Arl1 and Imh1 are required for the proper transport of Snc1 under Tunicamycin-induced UPR. Moreover, the N-terminal region of Imh1 is essential for restoring the plasma membrane localization of Snc1 under ER stress. Rab6/Ypt6 GTPase is known to regulate vesicle transport from endosome to the TGN via recruiting Golgi associated retrograde-protein (GARP) complex. We further show that Imh1 cooperates with GARP to assist the exocytosis of Snc1 upon the UPR induction. Thus, we propose a novel action of Arl1-Imh1 on establishing the environment at the TGN upon ER stress that is critical for the recycling of an exocytic SNARE.

P2182/B430

Vamp4 Is a New Factors Involved in Membrane Remodeling of Immature Secretory Granules during Maturation.

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Insulin secretory granules (ISGs) named as dense-core vesicles (DCVs), a group of distinguishing organelles in pancreatic β cells, are responsible for storage and secretion of insulin to maintain blood glucose homeostasis. Although the molecular mechanism of ISG exocytosis has been well characterized in the past few decades, the molecular mechanism of ISG maturation is largely unknown because of its high dynamics. In this study, we focused on the vesicle-associated membrane protein 4 (VAMP4) and demonstrated that it participated in the membrane remodeling during ISG maturation.

P2183/B431

Identification of an Arginine Permease Can1 as a Putative Cargo of Arl1 Small GTPase.

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Recycling transmembrane nutrient transporters at the cell surface is critical for nutrient uptake and responses to extracellular signals. Nutrient transporters can be internalized from the plasma membrane

and delivered to early endosomes upon stimulation, then selected and sorted for recycling to the Golgi and subsequent delivery back to the cell surface. ADP-ribosylation factor-like protein 1 (Arl1) is an important regulator of intracellular vesicular trafficking in organisms ranging from yeast to mammals. Yeast Arl1 is known to regulate the transport of the GPI-anchored protein Gas1 from the late Golgi to the plasma membrane, however, whether it is involved in recycling vesicle transport remains unclear. Here, we identified an arginine permease Can1 as a putative cargo of Arl1 in recycling from endosome to the late Golgi. It is known that reduced Can1 cell-surface expression levels display resistance to the toxic arginine analog canavanine. Canavanine resistance (*cvr*) would result from reduced canavanine uptake due to defects in cell-surface targeting or recycling of the Can1 permease. We found that Arl1 as well as its upstream regulator Arl3 function in Can1 recycling transport, however Arl1-GEF Syt1 and Arl1-effector golgin Imh1 are not involved in. We observed that in *arl1Δ*, Can1-GFP is mislocalization and co-localized with TGN marker Sec7-mRFP. Using sucrose gradient centrifugation analysis, we demonstrated that the subcellular distribution of Can1 is altered in *arl1Δ* cells. In addition, Arl1-related proteins, Cog8, Ypt6, Vps53, are also involved in Can1 transport, which were also observed in Canavanine resistance plating assay and cell localization analyses. Finally, flippase Drs2, a complex with Arl1, is also able to regulate transportation of Can1. Taken together, our results suggest that Arl1 has a role in regulating Can1 recycling from endosome to the Golgi.

P2184/B432

Action of Arl1 GTPase and Golgin Imh1 in Ypt6-independent Retrograde Transport from Endosomes to the *Trans*-Golgi Network.

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Action of Arl1 GTPase and golgin Imh1 in Ypt6-independent retrograde transport from endosomes to the trans-Golgi network the Arf and Rab/Ypt GTPases coordinately regulate membrane traffic and organelle structure. Studies and evidences have implied that the two families function in parallel; nevertheless the detailed mechanism between their complementation remained to be clarified. In this study, we found that Golgi-localized Arf, Arl1 and its effector, golgin Imh1, but not flippase Drs2 serve as high-copy suppressors of the defects in retrograde trafficking from endosome to *trans*-Golgi network (TGN) as well as high temperature growth retardation. Moreover, only Arl1 or Imh1, but not Arl3 or Syt1, interdependently restore the defects in ypt6-deleted cells. We further demonstrated that Arl1 and Imh1 reinforce the recruitment of the tethering factor, Golgi associated retrograde-protein (GARP) complex, to the TGN in the absence of Ypt6. To be more specific, the recruitment of GARP complex to TGN is essential for the suppression of the defects in ypt6-deleted cells. Interestingly, we found that the N-terminal domain of Imh1 is critical for restoring GARP complex localization and endosome-to-TGN transport in ypt6-deleted cells. Collectively, our study illustrates the mechanism underlying the suppression of Arl1 and Imh1 in endosome to TGN trafficking defects, revealing the involvement of GARP complex recruitment in the compensation for defects in dysfunctional Ypt6 conditions.

P2185/B433

α-Arrestins Regulation of Kir2.1 Potassium Channel Trafficking Depends on Select Trafficking Machinery in a Yeast Model System.

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Protein composition at the plasma membrane is tightly regulated, with rapid protein internalization and selective targeting to the cell surface in response to environmental changes. Ion channels are dynamically relocalized to or from the plasma membrane in response to physiological changes, allowing organisms to maintain osmotic and salt homeostasis. Critical to cardiac function is the localization of Kir2.1, an inward rectifying potassium channel, at the cell surface. Kir2.1 restores the resting membrane potential of heart cells after each contraction and readies the cell for the next action potential. Defective Kir2.1 trafficking and activity is associated with diseases, including Andersen-Tawil syndrome, highlighting the importance of understanding Kir2.1 trafficking. We used a yeast model system where the endogenous potassium channels were deleted and Kir2.1 is expressed; Kir2.1 is able to promote yeast growth on low potassium medium. Using this system, we discovered that specific α-arrestins, an emerging class of protein trafficking adaptors, regulate Kir2.1. Specifically, we found that the Ldb19/Art1, Aly1/Art6, and Aly2/Art3 α-arrestin adaptor proteins promote Kir2.1 trafficking to the cell surface, increasing intracellular potassium levels. To better quantify the intracellular and cell surface populations of Kir2.1, we created fluorescence-activating protein fusions and for the first time used this technique to quantify the cell surface residency of a plasma membrane protein in yeast. We now aim to elucidate the trafficking machinery needed for the α-arrestin-dependent trafficking of Kir2.1. We have identified a number of trafficking factors utilized by the α-arrestins to regulate Kir2.1. α-Arrestin Aly1 requires the AP-1 complex, a clathrin adaptor complex thought to recruit clathrin to vesicles shuttling between the endosomes and the Golgi, to promote Kir2.1-mediated growth on low potassium. Curiously, α-arrestins Aly2 and Ldb19 did not require AP-1 for their role in promoting Kir2.1-mediated growth suggesting that these α-arrestins operate in distinct pathways. Instead, α-arrestin Aly2 is dependent on Vrp1, a protein needed for actin network formation at endocytic sites, and Npr1, a nutrient responsive kinase downstream of TORC1 known to phosphorylate Aly2. Together, our findings show that the α-arrestins function in multiple distinct pathways while trafficking a common cargo in Kir2.1. These multiple inputs on a single cargo may allow for plasticity in the regulation of Kir2.1 trafficking to fine tune Kir2.1 activity at the cell surface.

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Mal2 Function in Basolateral Secretion Is Likely Regulated by Serine Phosphorylation and Actin Binding.

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The focus of our laboratory is identifying and defining the mechanisms that regulate polarized sorting of newly synthesized apical and basolateral resident proteins and secreted proteins. For the past 15 years, we have been investigating the role of the tetraspanning integral membrane protein, MAL2 (myelin and lymphocyte protein 2), in these processes. We first confirmed that MAL2 functions in the transcyotic sorting of newly synthesized apical residents. We further determined that MAL2 selectively regulates the TGN to basolateral sorting of newly synthesized polymeric IgA receptor, and more recently, we

determined that MAL2 participates with its known binding partner, serine/threonine kinase 16, in the regulation of constitutive basolateral albumin secretion. To identify the molecular determinants in MAL2 that confer specificity to its function in each of these pathways, we generated a panel of MAL2 mutants. Unlike other MAL family members, only MAL2 encodes the N-terminally-located, cytoplasmically oriented VPPPP and FPAP sequences that resemble the F/L/W/YPPPP recognition sites for EVH1 (enabled, VASP, homology1) motifs present in Ena/VASP proteins. We have changed each of the two motifs to alanines either alone or together. We have also generated mutants in the N-terminally located, cytoplasmically-oriented ser17. In this case the ser was replaced to an ala or asp (a phospho-mimetic). We started our analysis with basolateral secretion assays. Our preliminary results indicate that cells expressing the FPAP (but not VPPPP) mutants displayed decreased albumin release implying a link to the actin cytoskeleton in MAL2 function in secretion. We also determined that expression of the S17A mutant led to impaired secretion implying MAL2 phosphorylation is important for its role in secretion. We are currently testing these mutants in transcytosis and basolateral membrane protein delivery to uncover whether these motifs selectively confer specificity to the different MAL2-mediated trafficking pathways.

P2187/B435

Exocyst Conformational Changes Control Interactions with SNARE and Sec1/Munc18 Proteins.

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Membrane trafficking is an essential eukaryotic process necessary for a variety of cellular functions including, but not limited to, growth, cell division, motility, and signaling. The trafficking and fusion of cargo-containing vesicles with organelles and the plasma membrane is tightly regulated to ensure that proteins and lipids are delivered to their correct cellular destination. SNARE-mediated fusion is regulated by both Sec1/Munc18 (SM) proteins and multi-subunit tethering factors. The exocyst is a conserved, hetero-octameric tethering complex that is essential for exocytosis. Several of the exocyst subunits interact directly with SNAREs and the SM protein Sec1, however it remains unclear how the full complex coordinates these interactions to regulate SNARE complex formation and membrane fusion. Intriguingly, we found that the assembled exocyst octamer interacts only weakly with SNAREs and Sec1, despite previously observed robust SNARE/Sec1 binding to individual recombinant exocyst subunits. We purified exocyst subcomplexes (modules) and showed that a module of exocyst has increased apparent affinities for SNAREs and Sec1, suggesting that SNARE/SM binding sites are occluded within the full exocyst complex. As we cannot detect the presence of this module in wild-type yeast, we propose that the octameric complex undergoes regulated conformational changes to interact with SNAREs/Sec1. We demonstrate that gain-of-function mutations of the exocyst subunit Exo70, previously shown to rescue the *cdc42-6* allele and *rho3Δ*, "activate" the complex by stimulating SNARE binding without disassembling the complex. These activated exocyst complexes have an altered structure, as shown by negative stain electron microscopy, revealing that the SNARE/Sec1 binding subunit Sec6 is more dynamic and accessible than in the wild-type complex. We conclude that conformational changes of the exocyst complex regulate interactions with SNAREs and Sec1 to control SNARE-mediated membrane fusion during exocytosis.

P2188/B436

Tumor Protein D54 Defines a New Class of Intracellular Transport Vesicle.

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Transport of proteins and lipids from one membrane compartment to another is via intracellular vesicular carriers. Several classes of carrier have been described based on morphology, location and function. For example, clathrin-coated vesicles (50-100 nm diameter) formed at the plasma membrane or trans-Gogi network (TGN), COPII-coated vesicles (60-70 nm) originating at the endoplasmic reticulum (ER), and intra-Golgi transport vesicles (70to90 nm). Whether cell biologists have a complete inventory of all classes of transport vesicle is an interesting open question. We began by investigating the function of Tumor Protein D54 (TPD54/TPD52L2), a member of the TPD52-like family. TPD54 is one of the most abundant proteins in HeLa cells but its function is unclear. We found that TPD54 was involved in multiple membrane trafficking pathways: anterograde membrane traffic, recycling endocytosed proteins back to the cell surface and Golgi integrity. To understand how TPD54 controls these diverse functions, we used an inducible method to reroute TPD54 to mitochondria. Surprisingly, this manipulation resulted in the capture of many small vesicles (30 nm diameter) at the mitochondrial surface. Superresolution imaging confirmed the presence of similarly sized TPD54-positive structures under normal conditions. We demonstrated that INVs meet three criteria for vesicle functionality. They contain specific cargo, they have certain R-SNAREs for fusion, and they are endowed with a variety of Rab GTPases (16 out of 43 tested). We propose that TPD54 defines a new class of transport vesicle, which we term intracellular nanovesicles (INVs). Visible by EM, INVs are small and uncoated; likely escaping previous detection due to their inconspicuousness. The molecular heterogeneity of INVs and the diverse functions of TPD54 suggest that INVs have diverse membrane origins and are likely to have a number of destinations. We conclude that INVs are a generic class of transport vesicle which transfer cargo between these varied locations.

P2189/B437

The Transmembrane Domain of Single-spanning Transmembrane Proteins Contributes to Intra-Golgi Membrane Localization and Export.

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The Golgi complex is the central organelle of the secretory pathway, where secretory proteins are glycosylated and sorted for their export to other parts of the cell. Cargo glycosylation needs to follow a sequential order, achieved thanks to the polarized organization of the Golgi complex, formed by a stack of biochemically-distinct flat cisternae. However, how secretory cargoes visit first the membrane regions enriched in glycosylation enzymes before being exported is still not fully understood. Different protein retention mechanisms have been proposed, such as recognition of cytoplasmic motifs, protein oligomerization, or mechanisms based on the biophysical properties of the membrane. Amongst these, the hydrophobic matching hypothesis suggests that single-spanning transmembrane proteins preferentially partition into membranes with a thickness that matches the hydrophobic length of the

protein transmembrane domain (TMD). According to this hypothesis, Golgi-resident enzymes and secretory cargoes -due to their distinct TMD properties- segregate into membrane domains of different thickness, so-called processing and export domains, respectively. However, this hypothesis has not been fully tested for intra-Golgi protein localization. To test the contribution of the hydrophobic matching mechanism to the sorting of resident and secretory single-spanning transmembrane proteins at the Golgi membranes, we focused on the *trans*-Golgi enzyme sialyltransferase (ST), and TGN46, a protein that cycles between the *trans*-Golgi Network (TGN) and the plasma membrane. We first established by fluorescence confocal and super-resolution microscopy their localization in different regions of the *trans*-Golgi/TGN membranes. Since these proteins' TMD length and amino acid composition are different, we tested their involvement in intra-Golgi differential partitioning. Our results show that neither the length nor the amino acid composition of the TGN46 TMD dictates intra-Golgi localization or glycosylation status. Similarly, increasing the TMD length of ST does not alter its intra-Golgi localization. Moreover, our data suggests that the cytosolic domain of TGN46 is a signal for its intra-Golgi localization. However, adding this domain to ERGIC53, a protein that does not traffic beyond the *cis*-Golgi, is not sufficient to localize it to the TGN, suggesting that the TMD is also required for this purpose. Interestingly, we found that the TMD composition is important for the efficient export of TGN46, as shown by FLIP microscopy. Based on our findings, we suggest that the hydrophobic matching mechanism drives intra-Golgi protein localization together with other stronger signals, and it also contributes to the efficient export of secretory cargoes from the TGN.

P2190/B438

Measuring CD147 and MCT1 Dynamics with an ARE-containing Tetracycline Induction System.

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The transmembrane glycoprotein CD147 (Emmprin, Basigin, BSG) is highly expressed in many forms of cancer. Shortly after CD147 is synthesized in the endoplasmic reticulum, it interacts with members of the monocarboxylate transporter (MCT) family before being trafficked through the Golgi apparatus and trafficking vesicles on the way to the plasma membrane. It is established that CD147-MCT interaction is required in order for either CD147 or MCTs to be expressed at the plasma membrane. However, whether the complex created between MCTs and CD147 remains intact throughout the dynamics of trafficking, or if the complex is broken and the proteins act on their own remains unknown and is the aim of our study. Using fluorescence microscopy, we show that the steady-state localizations of CD147, MCT1, and a fusion of CD147-MCT1 all differ. CD147 reaches the plasma membrane at a moderate level with some internalized vesicles. MCT1 is predominantly localized to internal vesicles. CD147-MCT1 predominantly localizes to the plasma membrane. This suggests that the composition of the complex impacts its function. These observations were made as constitutively expressed fusion proteins in their steady state. To assess the dynamics of the proteins, it is necessary to quantify protein synthesis and decay. We developed a modified tetracycline-inducible expression system for studying protein decay that avoids high cost (e.g. SNAP tagging) or use of radioactivity. Since the mRNA decay from the tetracycline expression system is too slow to accurately measure protein decay, we modified the expression system to include an Adenylate-Uridylate Rich Element (ARE) from the Insulin-like Growth Factor (IGF) in its 3' untranslated region. By quantitative RT-PCR, we show that the decay of mRNA containing the IGF-ARE is very rapid which allows for a close approximation of protein decay. By western blotting, we show the decay of CD147 protein expressed individually versus a fusion of CD147-MCT1.

This system provides a tool for determining factors that contribute to the increased levels of CD147 found in cancer cells.

P2191/B439

Effects of Oxidative Stress on Expression of Arf Guanine Nucleotide-exchange Factors BIG1 and BIG2.

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Brefeldin a (BFA)-inhibited guanine nucleotide-exchange protein BIG1 and BIG2 that are encoded by the ADP-ribosylation factor guanine nucleotide-exchange factor (*Arfgef*)*1* and *Arfgef2* genes, respectively, were initially purified together in a multiprotein complex from bovine brain cytosol. These GEFs are 74% identical in amino acid sequences and with 90% identity in the Sec7 domains that responsible for ADP-ribosylation factor (Arf) activation. BIG1 and BIG2 accelerate the replacement of Arf-bound GDP with GTP to help the formation of intracellular vesicles for cargo transport between the Golgi, endosomal compartments, and the plasma membrane. It has been reported that BIG1 knockdown promotes glioblastoma U251 cell migration and down-regulation of BIG1 by miR-215 inhibited papillary thyroid cancer cell proliferation. The amount of migration-related molecules altered by the increase of HIF-1α expression under hypoxic stress is an essential event in the activation of cell motility. It remains unknown whether BIG1 and BIG2 could be regulated by hypoxia. Here, protein and mRNA levels of BIG1 and BIG2 were assessed in U251 cells treated with the hypoxic mimetic agents, DMOG and $CoCl_2$. We also generated the stably BIG1- or BIG2-knockdown cells by short hairpin RNA (shRNA) to investigate the functional roles of BIG1 or BIG2 in cell morphology, cell motility and cell proliferation. Taken together, our data provide information of effects of hypoxic stress on BIG1 and BIG2 expression and the roles of BIG1 and BIG2 on U251 cell migration and cell proliferation.

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Extracellular Vesicles

P2192/B440

Phluo_m153r-cd63, a Bright, Versatile Live Cell Reporter of Exosome Secretion and Uptake, Reveals Pathfinding Behavior of Migrating Cells.

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Small extracellular vesicles called exosomes affect a variety of autocrine and paracrine cellular phenotypes, including cellular migration, immune activation, and neuronal function. Understanding the function of exosomes requires a variety of tools, including live cell imaging. We previously constructed a live-cell reporter, pHluorin-CD63, that allows dynamic subcellular monitoring of exosome secretion in migrating and spreading cells. However, there were some caveats to its use, including dim fluorescence and the inability to make cell lines that stably express the protein. By incorporating a stabilizing mutation in the pHluorin moiety, M153R, pHluorin-CD63 now exhibits higher and stable expression in cells and superior monitoring of exosome secretion. Using this improved construct, we demonstrate visualization of secreted exosomes in 3D culture and in *vivo* and identify a role for exosomes in promoting leader-follower behavior in 2D and 3D migration. By incorporating a further non-pH-sensitive

red fluorescent tag, this reporter allows visualization of the exosome lifecycle, including multivesicular body (MVB) trafficking, MVB fusion, exosome uptake and endosome acidification. This new reporter will be a useful tool for understanding both autocrine and paracrine roles of exosomes.

P2193/B441

A Genetically Encoded Nanobody Reveals the Mechanism of Extracellular Vesicle Cargo Release in Cells.

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Cell to cell communication takes place through direct contact between cells and via secreted factors such as small molecular messengers and extracellular vesicles (EVs). EVs contain biomolecules such as proteins, lipids, and nucleic acids and mediate a myriad of cellular processes including regeneration, development, immune response and disease pathogenesis. They also show promise as drug delivery vehicles owing to their low immunogenicity, cell specificity, and efficient biomolecule delivery. In order to induce functional changes in recipient cells, EVs need to unload their contents. Currently, the mechanism of cytosolic delivery of EV cargo remains largely unknown. Using a cytosolic probe, i.e., a genetically encoded nanobody that specifically binds to EV cargo, combined with correlative light and electron microscopy (CLEM), we show that EV cargo is released from endosomes through EV-endosome membrane fusion. Specifically, cytosolic exposure of EV cargo was detected by nanobodies, resulting in the formation of nanobody punctae. Subsequent CLEM of the nanobody punctae revealed late endosomes/multivesicular bodies as the underlying cellular compartments from where cargo release takes place. Neutralization of endosomal pH and accumulation of endosomal cholesterol blocked cargo release, showing that EV cargo release is dependent on endosomal pH and cholesterol level. Furthermore, the absence of endosome permeabilization in EV-treated cells showed that membrane fusion is the mechanism behind cargo release. We show that genetically encoded nanobodies that recognize EV content offer an excellent approach to study both the mechanism and efficiency of EV cargo release in cells. Our findings provide a foundation for EV-mediated drug delivery improvement and EV-mediated disease transmission prevention by stimulating and inhibiting EV-endosome membrane fusion, respectively.

P2194/B442

Extracellular Vesicles from Differentiated Neuronal Cells Promote Neural Induction of Mesc through Cyclind1.

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Exosomes may contribute to a range of important biological processes by mediating the transfer of protein and RNA cargoes between cells. However, how exosomes function in developmental events such as in the differentiation and organization of nerve cells remains unclear. Here, we show dynamic changes in a physical property, the buoyant density, of extracellular vesicles secreted by PC12 cells stimulated with nerve growth factor (NGF) leading to neuronal differentiation. Exosomes secreted from differentiated PC12 cells and Neural 2A cells promote neural induction of mouse embryonic stem cells (mESCs). One protein that contributes to this induction is the cell cycle-related factor cyclinD1, which we find enriched within exosomes derived from differentiated neuronal cells. Exosomes purified from cells

overexpressing cyclinD1 were more potent in neural induction of mESC cells. Depletion of cyclinD1 from the exosomes reduced the neural induction effect. Our results suggest that extracellular vesicles regulate neural development through sorting of cyclinD1.

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Cell Polarity Establishment and Maintenance

P2195/B444

Anisotropic Cues Promote Symmetry Breaking to Initiate Migration of Adherent Cells.

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Symmetry breaking is the transition of cells from a symmetric to an asymmetric state. After symmetric spreading of adherent cells, symmetry breaking generates regions of protrusion and retraction, enabling cells to develop polarity and initiate migration. It is unclear if the timing of symmetry breaking is predominantly controlled by an internal timing mechanism, or whether it is responsive to external anisotropic cues such as confinement, substrate adhesiveness, and cell-cell contact. While these cues are known to affect the behaviors of post-symmetry breaking cells such as migration, their effects on symmetry breaking are less explored. By manipulating culture conditions or micropatterning the substrate, we show that NRK-52E epithelial cells and NIH 3T3 fibroblasts break symmetry at faster rates in response to anisotropic cues including cell-cell contacts, cell-substrate adhesions, and substrate stiffness. These cues may be located tens of microns away from the cell border. Furthermore, symmetry breaking may be promoted by asymmetric stimulation of either protrusion or retraction, with the former being more stimulative than the latter. Using pharmacological agents, we show that the promotion of symmetry breaking by anisotropic cues is dependent on the formin pathway, suggesting involvement of formin-dependent filopodia in probing the environment. While it is difficult to rule out an internal timing mechanism for symmetry breaking, our results suggest that mechanotransductive cues that regulate directed cell migration also provide effective stimulations for symmetry breaking, and that filopodia may play a central role of sensing for regulating both initial polarization and steady state migration.

P2196/B445

Competing Forces Redistribute Surface Macromolecules during EF-directed Cell Migration.

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DC electric fields (EFs) polarize cells, directing their migration and elongation. Cells sense and respond to EFs as weak as 10mV/mm, much weaker than EFs required to depolarize excitable cells. We propose that applied EFs polarize cells by redistributing plasma membrane macromolecules. Two competing forces in the plane of the plasma membrane control electromigration. Electrophoresis of surface macromolecules with net negative charge is toward the anode and electro-osmosis (electrically driven water flow) next to the negatively charged cell surface promotes water flow in the cellular boundary layer toward the cathode. We present a molecular flux model that predicts electromigration of membrane surface macromolecules based on the size and charge of the macromolecule, and the average surface charge of the cell. We have compared predictions of the flux model with the measured electromigration for the surface protein, tdTomato-GPI, under different EF strength and pH. We show that reduction of electro-osmosis with neutral, viscous polymers reverses the direction of migration

from cathode to the anode. We also describe a method for identifying the physical properties of plasma membrane proteins to predict the asymmetric redistribution of other surface macromolecules. These results are consistent with the model that applied EFs promote an asymmetric distribution of cell surface macromolecules while polarizing and directing cell migration during healing of chronic epidermal wounds and induction of cellular polarity during tissue engineering.

P2197/B446

BRCA1 Haploinsufficiency Results in Aberrant Mammary Epithelial Cell Polarity.

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BRCA1 mutation-bearing (B1+/-) women are more likely to develop breast and/or ovarian cancer than their B1+/+ counterparts. Many such cancers have lost all intact B1 function implying that a full loss of B1 function in these particular cancers is a pivotal step in B1 breast cancer development. But how B1 loss translates into breast cancer development remains incompletely understood. In particular, the early events that occur following loss of B1 function, which lead to BrCa development, remain unclear. We have found that human mammary epithelial cells (HMECs) obtained from B1 mutation carriers (B1 wt/mut genotype), which are competent for multiple B1 functions, show aberrant polarity when grown in 3D cell culture unlike HMECs taken from healthy women. These cells are also defective in 2D migration. The polarity defects of primary B1 wt/mut HMECs were reproducibly detected when the cells from patients carrying different clinically-relevant B1 mutations were analyzed. This suggests that these phenotypic abnormalities represent a novel B1 haploinsufficiency phenotype in human mammary cells. Our data also suggest the existence of a new cell biological link between BRCA1 and the cytoskeletal cell motility machinery. Collectively, our work reveals a new haploinsufficient role for BRCA1 in maintaining normal mammary epithelial cell biology. A relevant question is whether this function is of clinical importance.

P2198/B447

Nuclear and Cytoskeleton Polarization in Differentiating Ipsc Derived Retinalpigmentepithelium.

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The inherent capacity of Pluripotent Stem Cells (PSCs) to differentiate into any cell type combined with the reporter Induced Pluripotent Stem Cell (iPSC) technology to tag endogenous proteins linked to the cytoskeleton, nuclear lamina or any other organelle with GFP allows visualization of cellular architecture remodeling during the differentiation process. We developed a protocol to differentiate iPSCs into fully polarized and functional retinal Pigment Epithelium (RPE) cells. This developmentally guided differentiation of iPSCs into RPE recapitulates the embryonic stages of RPE development from a proliferating neuroectoderm cell into post-mitotic RPE cell. Differentiation of RPE from reporter iPSCs with GFP-tagged nucleus and cytoskeleton proteins have allowed us to discover how RPE cells acquire polarization. Embryonically, RPE is of neuroectoderm origin. We used dual SMAD inhibition to generate neuroectoderm, directed the differentiation of neuroectoderm cells into RPE progenitors by inhibiting the FGF and TGF pathways, and addition of Activin-A induced the fate of RPE progenitors into the committed RPE cells. Cytoskeleton organization in a specific order is needed to attain complete polarization and hexagonal morphology of RPE cell in a monolayer. We used GFP tagged TUBA1B and

LAMINB iPSC lines from Allen Institute to determine changes in the arrangement of the cytoskeleton (tubulin filaments) and nuclear lamina during differentiation into RPE. iPSC cells were seeded on iBidi chamber slides for differentiation, fixed after the end of the early neuro-ectoderm phase, progenitor middle phase, and final RPE committed phase, stained for the early and late RPE specific markers and imaged using an LSM700 microscope. The expression levels of PAX6 and MITF, the RPE specific transcription factors determine the differentiation of neurectoderm to RPE progenitor cells. This work has provided an improved understanding of RPE polarization during differentiation from iPSCs.

P2199/B448

Shear Stress Modulation of Bmp/alk1 Signaling and Flow-polarization in Endothelial Cells Revealed by an All-in-one Multi-shear Stress Microfluidic Device.

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Vascular morphogenesis is driven by proliferation and collective movement of endothelial cells (ECs). A hierarchically organized vascular network emerges during angiogenesis, and matures as blood vessels remodel. Defects in angiogenesis and remodeling, via overgrowth or inadequate network elaboration, lead to various diseases. To understand the origin of these defects we seek to understand how ECs respond to biomechanical and biochemical cues in their microenvironment. We focus on the effect of bone morphogenetic protein 9 (BMP9) since it has been shown to interact with the cellular receptor ALK1 to polarize EC cells, modulate EC migration, and direct vascular morphogenesis. To understand how flow and BMP9 interact we developed a microfluidic device together with a custom image analysis pipeline to quantify end-point immunofluorescence staining. Our device and flow protocols bracket physiological wall shear stress (SS) conditions in the artery environment to expose how ECs respond to both SS and growth factor stimulation. We named our microfluidic system "All-in-One" since it is capable of delivering multiple levels of laminar and gradient SS in different regions of the same device, so responses to biomechanical cues could be evaluated easily. To modulate biochemical cues we cultured confluent HUVECs in BMP-depleted serum for 4 hours, and then either added 2% FBS or kept ECs in BMP-depleted serum for 1 hour. After 1 hour of flow we observed pSMAD1/5/9 nuclear translocation and by 24 hours we found ECs aligned to the direction of the flow. When SS and ligands stimulated ALK1 signaling together, pSMAD translocation increased synergistically. Under conditions of reduced BMP9, nuclear translocation of pSMAD is strongly reduced. By comparing pSMAD translocation across many flow conditions, we find flow increased translocation but that the level of translocation was independent of the flow magnitude. All-in-One also allows quantification of cell polarity, defined as the vector from the center of the nucleus to the center of the Golgi apparatus. SS stimulation decreased the percentage of downstream-polarized ECs dependent on SS-magnitude (24±9% to 15±11%, $p < 0.05$ with t-test) while ligands increased the portion of upstream-polarized ECs (22±10% to 29±15%, $p < 0.05$ with t-test). Both high SS and serum stimulation reduced downstream-polarization and expanded the population of upstream-polarized ECs. Interestingly, the magnitude of SS has differing impact on ALK1 signaling and flow-aligned EC polarity; while flow-alignment depends on SS-magnitude, ALK1 signaling did not. To better understand the role ALK1 signaling play in EC migration, we plan to extend our microfluidic study to include high-resolution imaging of live ECs under spatially varying SS and BMP9 cues.

P2200/B449

Apico-basolateral Symmetry Breaking in the *C. Elegans* Intestinal Epithelium.

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Cells polarize by adopting spatially distinct structures and is an important process for cell and tissue function. One type of polarized cells is the epithelial cells that line tissues, which have an apical surface facing exteriorly or into a lumen and basolateral surfaces oriented into the body. During development, non-polarized precursors reproducibly define the location of this apico-basolateral axis in a process called symmetry breaking. Despite its requirement for polarization, epithelial symmetry breaking remains poorly understood, especially in *vivo*. To elucidate symmetry breaking mechanisms we are studying the *C. elegans* embryonic intestine, a simple epithelial tube composed of 20 'E' cells that orient their apical surfaces toward a common midline surrounded by basolateral surfaces facing each other and non-E cells. In this tissue, apical surfaces form at homotypic contacts and basal surfaces form at heterotypic contacts, thus we hypothesize that these differences in cell contact inform symmetry breaking. To test this hypothesis, we are using *pop-1* mutant embryos which produce twice as many E cells to characterize the distribution of polarity markers when there are ectopic E contacts. The intestines in these embryos are often arranged into multiple layers such that some E cells participate in homotypic contacts at all surfaces. The apical marker PAR-6 localizes to these exogenous homotypic contacts, consistent with a role for cell contact in symmetry breaking. While the molecular cues underlying this contact recognition remain unknown, likely candidates include adhesion proteins which could bind homophilically between E cells. For example, the two adhesion proteins HMR-1/E-cadherin and SAX-7/L1CAM are expressed in E cells and localize to cell membranes prior to polarization. Either protein alone is dispensable for polarity establishment, however the depletion of HMR-1/E-cadherin and SAX-7/L1CAM together results in a polarity defect, consistent with an early role for adhesion proteins in polarization. This work will highlight the role of cell/cell adhesion in *C. elegans* intestinal symmetry breaking, a mechanism likely conserved in other polarization contexts.

P2201/B450

A High-Content and Computational Approach to Study Cell Shape and Polarity in Epithelia.

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Epithelial cells have specialized structural features that are strictly linked to their functions. The Retinal Pigment Epithelium (RPE) is a monolayer of epithelial cells located in the back of the eye that constitutes part of the outer blood-retina barrier and plays a crucial role in photoreceptor survival. The integrity of this barrier is achieved by RPE cells through the establishment of epithelial phenotype - formation of tight junctions between neighboring cells allowing the cells to acquire apical-basal polarity. Failure in acquiring or maintaining the epithelial phenotype leads to retinal diseases, such as age-related macular degeneration (AMD). The aim of this study is to understand how the epithelial phenotype is established in RPE cells using high-resolution and high-content imaging. This is achieved using several CRISPR-modified induced pluripotent stem cell (iPSC) reporter lines generated by the Allen Institute (Seattle, WA), each expressing fluorescently tagged proteins to label intracellular organelles and structures. Among these are: cell membrane, nuclear envelope, Golgi apparatus, endoplasmic reticulum, mitochondria, lysosomes, endosomes, peroxisomes, tubulin and actin cytoskeleton, cell junctions and

focal adhesions. All these iPSC lines have been successfully differentiated into fully polarized RPE and imaged with high-resolution and high-content confocal microscopy. As a control, RPE has been treated with HPI-4, a drug known to disrupt the polarization process. A machine learning algorithm is used to automatically segment each labelled structure. More than 10,000 images of RPE cells are taken at every time point for each sample during the six weeks of RPE polarization. The location of organelles is precisely calculated by computationally averaging the data. A virtual 3D map of RPE representing the precise subcellular location of every organelle is being generated for each time point, showing how cellular structures rearrange themselves during maturation to establish the epithelial phenotype. In conclusion, these results shed light on the mechanisms responsible for impairment of polarity in RPE and, in general, other epithelia. Moreover, the method of investigation proposed here represents an innovative approach to describe morphology of intracellular structures and can be broadly applied.

P2202/B451

Asymmetric Localization of Dishevelled Protein Assemblies Via Lysosome Association during Oocyte Meiosis.

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Cellular asymmetries in the oocyte are intimately linked to the body axes formed during embryogenesis. Our goal is to understand how symmetry is broken in the oocyte to establish polarity. Here, we report an essential symmetry-breaking event that occurs during meiosis in oocytes of the sea star *Patiria miniata*. Using 4D time-lapse imaging and functional approaches, we find that the Wnt signaling component Dishevelled is uniformly distributed in the mature oocyte, but becomes specifically enriched at the vegetal cortex during meiosis. Localized Dishevelled is required to direct specification of the an terior-Posterior axis during early embryogenesis. Vegetal localization requires intrinsic cues from the oocyte cortex, as well as conserved domains within the Dishevelled protein that confer specific and separable granule forming and localization behaviors. We find that Dishevelled forms distinct populations of both dynamic and static granular assemblies that are locally concentrated on the cytoplasmic surface of lysosomes and at the cortex. Our results suggest a paradigm that bridges previous models for Dishevelled localization that invoke lipid binding versus accumulation in biomolecular condensates through phase separation.

P2203/B452

Tuba Regulates Cdc42 Functions in Neuronal Polarization Coupling Membrane and Actin Dynamics.

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Neurons are highly polarized cells, in which the sprouting and elongation of neurites, and its subsequent development in axons and dendrites, are key events during early neuronal differentiation. These processes depend mainly on changes in cytoskeletal dynamics and directed membrane traffic and are controlled by the Rho and Rab families of small GTPases respectively. The small Rho GTPase, Cdc42, has emerged as a critical regulator of neuronal polarity, however, to date, the GEF responsible of Cdc42 activation during polarity acquisition is unknown. In this work we proposed that Tuba is the GEF that activates Cdc42 during axon specification. Using primary cultures of rodent neurons, we observed a

sustained increase in Tuba expression during the initial stages of culture, concurring with polarity development. In addition, the overexpression of Tuba induces the formation of neurons with multiple axons, similarly to the phenotype observed with the expression of Cdc42 fast cycling. On the other hand, the loss-of- function of Tuba (mediated by the expression of an shRNA or a GEF-deficient form) impaired axon formation. Moreover, Tuba co-localizes with Rab8, a Rab GTPase involved in exocytic traffic from the trans Golgi network to the plasma membrane during neuronal polarization. In neurons, Rab8a dramatically alters normal neuronal polarization, because a dominant-negative form of Rab8a impaired axon formation, while a constitutively active form of Rab8a induces the formation of multiple axons. Accordingly, the overexpression of Rab8 induces an increase in the total activity of Cdc42 and this effects is mediated by Tuba. These findings suggest that Tuba acts as linker between the regulation of cytoskeleton dynamics and the directed vesicular traffic during neuronal polarization.

P2204/B453

Cdc42 GEFs Gef1 and Scd1 Crosstalk to Promote Transition from Monopolar to Bipolar Growth in Fission Yeast.

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The GTPase Cdc42 is a major regulator of cell polarity in most eukaryotes. Cells polarize when they break symmetry by establishing a Cdc42-activating positive feedback loop. This leads to accumulation of Cdc42 regulators at one site at the expense of other sites, resulting in a single growing pole. How, then, do cells activate growth at additional sites in the presence of an established growing pole? to address this, we used fission yeast, which grow in a monopolar manner after cell division and eventually transition to bipolar growth. A previous model proposes that bipolar growth occurs once cells reach a threshold size at which they synthesize sufficient activators for two growth sites. However, cell cycle mutants (*cdc10*) that remain monopolar can achieve a large cell size, indicating that a size threshold alone does not lead to bipolarity. In fission yeast, positive feedback is established first at the pre-existing, dominant old-end. Bipolar growth occurs when a new-end can compete with the old-end to establish a second site of Cdc42-activating positive feedback by an unknown mechanism. Cdc42 is activated by the GEFs Gef1 and Scd1. We find that in *gef1Δ* mutants, cells fail to establish a second site of growth since they cannot overcome the dominance at the old-end. Moreover, in these mutants Scd1 is monopolar, suggesting that Gef1 regulates Scd1 localization. Investigating the relationship between Gef1 and Scd1 is difficult due to the dynamicity of the GEFs at the cell ends. We took advantage of our previous finding that Gef1 and Scd1 sequentially localize to the cell division site during cytokinesis. Using cytokinesis as a paradigm, we find that Gef1 promotes Scd1 localization via Cdc42 activation. Our data show that Gef1-mediated active Cdc42 recruits the scaffold protein Scd2 which then recruits Scd1. This regulation also occurs during interphase at the new-end where Gef1 activates Cdc42 to provide a seed for Scd1/Scd2-mediated positive feedback to overcome old-end dominance. Furthermore, we find that Gef1 localization itself is tightly regulated, with Scd1 restricting Gef1 to sites of polarized growth to maintain cell shape and prevent ectopic growth sites. Our data reveal an elegant regulatory pattern in which Gef1 enables a new site to overcome old-end dominance by establishing a second site of Scd1-mediated Cdc42 activity to promote bipolar growth.

P2205/B454

Cell-Cycle-Dependent Cues Temporally Regulate Cdc42 Activity at Growth Sites in Fission Yeast.

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Cdc42 is the master regulator of polarized cell growth in most eukaryotes. In fission yeast, Cdc42 activity, and thus growth, gradually stops at the cell ends upon mitotic entry and resumes after the G1/S transition once cytokinesis completes and cells abscise. It is unknown how Cdc42 is inactivated at the cell ends during mitosis and whether resumption of Cdc42 activity requires completion of cytokinesis. To investigate the cell-cycle-dependency of Cdc42 activity at the cell ends, we have developed a system whereby we can delay cytokinesis while mitosis progresses via a temporary Latrunculin a (LatA) treatment. In these cells, Cdc42 activation resumes at the ends even before the completion of cytokinesis. We call this the PrESS phenotype - polar elongation sans cell separation. This indicates that Cdc42 activation at the cell ends is independent of completion of cytokinesis. Mitotic exit requires degradation of the G2/M cyclin Cdc13. In cells arrested in mitosis due to expression of a non-degradable *cdc13*, the frequency of the PrESS phenotype decreases. Moreover, G1/S-cyclin mutants, which have a lengthened G1 cell cycle phase, display an enhanced PrESS phenotype. This suggests that Cdc42 activity at the cell ends is inhibited by an active Cdk1/Cdc2-Cdc13 complex and can resume only after entry into G1. Next, to identify how Cdc42 is regulated at the cell ends during mitosis, we analyzed the PrESS phenotype in mutants of different Cdc42 regulators. Cdc42 is inactivated by the GTPase activating proteins (GAPs) Rga4 and Rga6. We find that loss of *rga4* or *rga6* increases the frequency of the PrESS phenotype. Furthermore, Rga4 and Rga6 show enhanced localization at the cell ends during mitosis. We also find that the scaffold Scd2, which is required for polarized Cdc42 activation, remains at the cell ends even during mitosis. This suggests that loss of Cdc42 activity at the cell ends during mitosis is likely not due to the inability to recruit Cdc42 activators, but rather due to enhanced Rga4 and Rga6 localization. Based on these data, we propose that the cell cycle temporally modulates Cdc42 activity at the cell ends via regulation of the GAPs such that growth does not occur during mitosis.

P2206/B455

Proteomic an alysis Uncovers Distinct Segregation Patterns of Yeast Cell-peripheral Proteins.

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Protein segregation contributes to various cellular processes such as polarization, differentiation, and aging. However, the difficulty in global determination of protein segregation hampers our understanding of its mechanisms and physiological roles. To overcome this, we globally monitored segregation of preexisting proteins during cell division of budding yeast, and identified crucial domains that determine the segregation of cell-peripheral proteins. Remarkably, the proteomic and subsequent microscopic analyses demonstrated that the flow through the bud neck of the proteins that harbor both endoplasmic reticulum (ER) membrane-spanning and plasma membrane (PM)-binding domains is not restricted by the previously suggested ER membrane or PM diffusion barriers but by septin-mediated partitioning of the PM-associated ER (pmaER). Furthermore, the proteomic analysis revealed that although the PM-spanning t-SNARE Sso2 was retained in mother cells, its paralog Sso1 unexpectedly showed symmetric localization. We found that the transport of Sso1 to buds enhances cell elongation during pseudohyphal growth. Taken together, these data resolve long-standing questions about septin-

mediated compartmentalization of the cell periphery, and provide new mechanistic insights into the segregation of cell-periphery proteins and their cellular functions.

P2207/B456

Patterning of Membrane-associated Proteins through Membrane Flows.

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How cells pattern and polarize in response to internal and external cues is a fundamental biological problem. In the rod-shaped fission yeast cell, the polar and lateral plasma membranes are distinct in lipid and protein composition. Polar zones are defined by local Cdc42 GTPase activity, which directs polarized secretion. By contrast, how the lateral membrane is patterned and how membrane-associated proteins, such as the Cdc42 GAP Rga4, accumulate laterally is less well understood. We have recently developed a light-inducible tool based on the CRY2PHR-CIBN system to acutely recruit proteins to the plasma membrane upon blue light photo-stimulation. Blue light activation triggers the formation of large CRY2PHR-CIBN complexes at the cell cortex, which are initially homogeneously distributed. Remarkably, after photo-activation, CRY2PHR-CIBN complexes are rapidly cleared from the growing pole(s). We hypothesize that exocytosis at the center of a broader zone of endocytosis at sites of growth acts as a driver of membrane flows causing this cell patterning. Mathematical simulations indeed show that the density of plasma membrane-associated proteins at the pole decreases according to membrane affinity (Koff). Experiments confirm a concentrated zone of exocytosis within a broader zone of endocytosis at zones of future CRY2PHR-CIBN clearance and disruption of the phenomenon by blocking polarized secretion. Empirical modulation of membrane binding affinity using both synthetic probes and the endogenous Rga4 further demonstrate that this parameter defines their cortical distribution. These results suggest that membrane flows created by the specific location of polarized secretion and endocytosis pattern the lateral distribution of membrane-associated proteins.

P2208/B457

Optogenetics Reveals Cdc42 Local Activation by Scaffold-mediated Positive Feedback and Ras GTPase.

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The small GTPase Cdc42 is critical for cell polarization. Scaffold-mediated positive feedback regulation was proposed to mediate symmetry-breaking to a single active zone in budding yeast cells. In rod-shaped fission yeast *S. pombe* cells, active Cdc42-GTP localizes to both cell poles, where it promotes bipolar growth. Here, we implement the CRY2-CIBN optogenetic system for acute light-dependent protein recruitment to the plasma membrane, which allowed to directly demonstrate positive feedback. Indeed, optogenetic recruitment of constitutively active Cdc42 leads to co-recruitment of the guanine nucleotide exchange factor (GEF) activator Scd1, in a manner dependent on the scaffold protein Scd2. We show that Scd2 scaffold function is completely bypassed and positive feedback restored by an engineered interaction between the GEF and a Cdc42 effector, the Pak1 kinase. Remarkably, such re-wired cells are viable and grow in a bipolar manner even when lacking otherwise essential Cdc42 activators. These cells also reveal that Ras1 GTPase plays a dual role in localizing and activating the GEF, thus potentiating the feedback. We conclude that scaffold-mediated positive feedback, gated by Ras activity, is minimally required for rod-shape formation.

P2209/B458

Role of Cdc42 GTPase-activating Proteins in Asymmetric Cell Division and Cdc42 Oscillatory Dynamics.

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The highly conserved small GTPase Cdc42 regulates polarized cell growth and morphogenesis from yeast to humans. We previously reported that Cdc42 activation exhibits oscillatory dynamics in *Schizosaccharomyces pombe* cells. Mathematical modeling suggests that this dynamic behavior enables a variety of symmetric and asymmetric Cdc42 distributions to coexist in cell populations. Individual growing wild type cells, however, follow a stereotypical pattern, where Cdc42 distribution is initially asymmetrical in young daughter cells, and becomes more symmetrical, enabling bipolar growth activation, as cell volume increases. To further explore whether different states of Cdc42 activation are possible in a biological context, we examined *S. pombe rga4Δ* mutant cells, lacking the Cdc42 GTPase activating protein (GAP) Rga4. We find experimentally that monopolar *rga4Δ* mother cells divide asymmetrically, leading to the emergence of both symmetric and asymmetric Cdc42 distributions in *rga4Δ* daughter cells. By genetic screening approaches to identify mutants that alter the *rga4Δ* phenotype, we tested the predictions of different computational models that reproduce the unequal fate of daughter cells. We find experimentally that the unequal distribution of active Cdc42 GTPase in daughter cells is consistent with an unequal inheritance of another Cdc42 GAP, Rga6, in the two daughter cells. Our findings highlight the crucial role of Cdc42 GAP protein localization in determining the morphological fate of cell progeny and ensuring consistent Cdc42 activation and growth patterns across generations.

P2210/B459

A Minimal System to Reconstitute Pom1 Gradient.

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A number of protein concentration patters have been reported to influence various process in Biology. One such pattern is Pom1 gradient found in fission yeast cells, S. Pombe., with maximum at the cellular poles. Pom1 gradient has been suggested to correlate cell size with cell division by regulating the activity of Cdr2 nodes at the cortex in the middle of the cell. SAD kinase Cdr2 has inhibitory effect on cyclin dependent kinase Cdk1 via Wee1 and hence can control mitotic entry of the cell. When the Pombe cells reach their characteristic length the gradient profiles from the two cellular poles do not overlap with the Cdr2 nodes and the fission yeast cell can undergo mitosis. Using the bottom up approach, we are trying to reconstitute Pom1 gradient in cell size confinements. Microfluidics is used to make cell size confinements and microtubule based transport is used to transfer proteins to the two poles in the confinement. Here we show how shape of the emulsion droplets influence the microtubule growth and forces them to grow along the longer axis in elongated droplets. We also establish microtubule plus end tracking inside the elongated droplets to enable protein transport. Combining the two results together, we aim to develop minimal system to reconstitute Pom1 gradient inside the emulsion droplets of cellular dimensions.

P2211/B460

Courtship Is a Two-way Conversation: Yeast Mating as a Model of Cell-cell Communication.

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Many cells track chemical signals during feeding, fertilization, development, and immune responses, but the mechanisms of target location are elusive. The budding yeast, an established model of such cell-cell communication, tracks a pheromone signal to locate a mating partner. Pheromone binding to cell surface receptors triggers the concentration of polarity proteins at a small zone of the cell cortex (the "polarity patch"). The polarity patch is oriented toward the partner, and through its effectors, directs polarized growth toward that mate. In classical models of polarization, a cell first decodes the pheromone gradient and then polarizes toward the region of highest signal. However, studies of yeast in artificial gradients found that cells that polarize in the wrong direction can move the patch to align with the partner. As receptors and G proteins concentrate at the patch, local pheromone sensing and secretion may mediate communication between partners. A fundamentally different "exploratory polarization" paradigm for orientation in a gradient posits that polarity sites move around the cortex in a search process, and that when the patches of two partner cells are aligned, each cell senses a high pheromone signal, causing the patches to stop moving. Unlike the artificial gradient studies, cells in mating mixes first assemble weak and transient polarity clusters that move erratically around the cell cortex, often engaging different partners before stably polarizing. It is unclear whether such polarity clusters are functionally important for partner identification. Here we ask whether the weak early clusters are engaged in exploratory polarization, only stabilizing when two clusters co-oriented, or if the cells perform a more classical global survey of the pheromone landscape, without need for the weak polarity clusters. To distinguish between these models, we performed live-cell fluorescence microscopy of mating mixes in which wildtype cells were paired with partners that either failed to make polarity clusters, or that made but could not stabilize clusters. Strikingly, we found that the wildtype cells displayed extended indecisive behavior and did not commit to such partners. Stabilizing the patches of polarity mutants with exogenous pheromone allowed wildtype cells to commit to them. We also confirmed that both sensing, signaling, and secretion markers were enriched at indecisive phase polarity clusters, thereby providing direct experimental evidence that local pheromone secretion promotes commitment to a partner, in support of the exploratory polarization model. We conclude that reciprocal communication between two partners' polarity sites is essential for successful commitment to a mating partner.

P2212/B461

Mechanical Feedback Maintains Polarization in Budding Yeast Mating Projection Growth.

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Cell Polarization is one of the commonly studied cases of spontaneous symmetry breaking in cells and controls many cellular processes, including morphogenesis, in budding yeast. However, the mechanisms that coordinate continued polarization to the growth region during mating projection formation, and the subsequent change in geometry from a spherical cell, remain unknown. We theoretically show that a genetically-encoded mechanical feedback relaying information about cell's geometry is sufficient to

maintain key polarity molecules and growth machinery remain localized to the site of growth. We have demonstrated that this same feedback mechanism also results in cell wall stability.

P2213/B462

Kar9 Symmetry Breaking Alone Is Insufficient for Spindle Positioning in Budding Yeast.

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Asymmetric cell division requires the alignment of the spindle along the polarity axis of the cell. The interactions of astral microtubule +ends with the cortex are needed for spindle positioning. Budding yeast has an asymmetric cell morphology, and the spindle must be correctly positioned at the bud neck and aligned to the polarity axis for a successful mitosis. Prior to anaphase, the spindle is positioned at the bud neck by Kar9 - a microtubule +end binding protein that binds the type V myosin Myo2. The interaction between Kar9 and Myo2 couples microtubule +ends to cortical actin in the bud, effectively aligning the entire spindle along the polarity axis. The asymmetric localization of Kar9 to one spindle pole is thought to be sufficient for spindle positioning. However, the temporal correlation between Kar9 symmetry breaking in relation to the alignment of the spindle has not been investigated. In this study, we develop an algorithm to quantitatively and reliably determine Kar9 symmetry breaking in a dynamic state and correlate it with stable spindle alignment (“perfect alignment”). Using confocal microscopy, we examine wild type cells, as well as cells expressing a Kar9 mutation (Kar9-AA) that inhibits its phosphorylation by Cdk1 and thus increases Kar9 association with both spindle poles. Strikingly, we find that Kar9 asymmetry is insufficient for perfect alignment, as the majority of asymmetric cells failed to display perfect alignment. Additionally, a subset of symmetric cells displayed perfect alignment, despite persistent attachment of Kar9 to both poles. Furthermore, we show that perfect alignment is correlated with Kar9 bud entry and persistence in the bud. We speculate that the binding of Kar9 to Myo2 is regulated in the bud. Based on these findings, we propose a revised model of spindle alignment, for which both Kar9 symmetry breaking and Kar9-Myo2 interactions are independently acting to align the spindle.

P2214/B463

The Role of Rna Structure in the Physical State and Maturation of Phase Separated Droplets.

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Analogous to the separation of oil from water, a process called liquid-liquid phase separation (LLPS) partitions cellular contents. LLPS is dictated by intrinsic biophysical/biochemical properties of component molecules. Many phase-separated droplets form from RNA-binding proteins in complex with target RNAs (RNPs). In pathological contexts, the dynamics of these molecules are disrupted and it is hypothesized this influences disease pathology. It is now appreciated that RNA sequence/structure, can dictate assembly and physical state of droplets. This provides a new role for RNA sequence, which is encoding mesoscale biophysical properties of cellular bodies. Although there is a link between RNA sequence and droplet behaviors, the details are still a mystery. We hypothesize that RNA serves as a scaffold for RNA-based condensates and that depending on the RNA sequences engaged in LLPS, droplets emerge with distinct biophysical properties and cellular functions. Specifically, we predict that sequence-directed RNA structures control inter-molecular RNA-RNA interactions and how RNA-binding proteins bind to RNAs in condensates. We previously found that the mRNA encoding the cyclin *CLN3* and the RNA-binding protein Whi3 condense to localize cyclin transcripts near nuclei in multinucleate fungal

cells. The structure of Whi3's target RNA, *CLN3,* is known to regulate features of droplet formation. In order to identify *CLN3* RNA sequences and structures important for Whi3 phase separation behaviors, we first employed a computational approach, which combines RNA structure predictions, RNA structure chemical probing data, and analysis of sequence/structural conservation across fungal species. This led to the identification of a number of RNA sequences/structures which regulate RNP droplet formation. Mutants were generated based on these predictions and these mutant *CLN3* RNAs were analyzed using an in *vitro* phase separation assay. Interestingly, one mutant which includes disruption of a potential site of intramolecular RNA/RNA pairing, an RNA structure that regulates protein binding site clustering, and protein binding site occupancy showed dramatic differences in droplet formation. Droplets made from mutant RNAs had altered biophysical properties (size, viscosity, diffusivity) suggesting that RNA-RNA and RNA-protein interactions are critical for the physical parameters of droplets. Current work is focused on testing the impact of these mutations in cells. These studies highlight the critical importance of specific RNA structures in determining key features of biomolecular condensates.

Neuronal Degeneration and Regeneration 2

P2215/B465

Neuronal Sphk1 Acetylates Cox2 and Contributes to Pathogenesis in a Model of Alzheimer'S Disease.

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Although many reports have revealed the importance of defective microglia-mediated amyloid β phagocytosis in Alzheimer's disease (AD), the underlying mechanism remains to be explored. Here we demonstrate that neurons in the brains of patients with AD and AD mice show reduction of sphingosine kinase1 (SphK1), leading to defective microglial phagocytosis and dysfunction of inflammation resolution due to decreased secretion of specialized proresolving mediators (SPMs). Elevation of SphK1 increased SPMs secretion, especially 15-R-Lipoxin A4, by promoting acetylation of serine residue 565 (S565) of cyclooxygenase2 (COX2) using acetyl-CoA, resulting in improvement of AD-like pathology in APP/PS1 mice. In contrast, conditional SphK1 deficiency in neurons led to reduction of SPMs secretion and abnormal phagocytosis similar to AD. Overall, these results reveal a novel mechanism of SphK1 pathogenesis in AD that leads to defective microglial phagocytosis due to impaired SPMs secretion, and suggests that SphK1 in neurons has acetyl-CoA dependent cytoplasmic acetyltransferase activity towards COX2.

P2216/B466

Genetic Ablation of Neuronal Mitochondrial Calcium Uptake Prevents Alzheimer'S Disease Progression.

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Background. Alzheimer's disease (AD) is characterized by extracellular deposition of amyloid beta, intracellular neurofibrillary tangles, synaptic dysfunction and neuronal cell death. These phenotypes correlate and are linked with elevated neuronal intracellular calcium ($_{i}Ca^{2+}$) levels. Recently, our group reported that $_{m}Ca^{2+}$ overload due to loss of $_{m}Ca^{2+}$ efflux contributes to AD progression. In this report we also noted proteomic remodeling of the mitochondrial calcium uniporter channel (mtCU). The mtCU is

the primary mechanism for Ca^{2+} uptake into the mitochondrial matrix, therefore, inhibition of the mtCU has the potential to reduce or prevent $_mCa^{2+}$ overload in AD. **Methods. To** define the role of $_mCa^{2+}$ uptake in AD we generated 3xTg-AD mutant mice with neuronal-specific deletion (Camk2a-Cre) of the mtCU pore-forming subunit, *Mcu*, and evaluated age-associated changes in cognitive function, and neuropathology. Neuroblastoma (N2a) and AD-mutant (APPswe) cell lines transduced with lentivirus encoding shRNA targeting MCU were used to examine mitochondrial calcium signaling, cell death and oxidative stress. **Results.** We observed that neuronal-specific loss of $_mCa^{2+}$ uptake in 3xTg-AD mice reduced Aβ and tau-pathology, synaptic dysfunction and cognitive decline. Knockdown of MCU in APPswe cell lines significantly decreased matrix Ca^{2+} content, oxidative stress and cell death. **Conclusions.** Inhibition of neuronal $_mCa^{2+}$ uptake represents a novel therapeutic target to reduce AD progression and associated mitochondrial dysfunction.

P2217/B467

Preventive Ex Vivo Gene Therapy for Stroke in Rat Model.

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Contemporary gene and cell technologies are widely employed in pre-clinical trials for treatment of different pathological conditions in appropriate animal models. The discovery of such new approaches for stroke therapy is under intensive investigation as well. Recently we have shown that intrathecal injection of genetically engineered human umbilical cord blood mononuclear cells (UCB-MC) transduced with adenoviral vectors (Ad5) which carried genes encoding vascular endothelial growth factor (VEGF), glial cell line-derived neurotrophic factor (GDNF) and neural cell adhesion molecule (NCAM) successfully rescued neurons following cerebral blood vessel occlusion. In the present study we employed the same gene-cell construct for preventive ex vivo gene therapy. Rats were anesthetized and intrathecally infused with 2×10^6 UCB-MC+Ad5-VEGF+Ad5-GDNF+Ad5-NCAM in 20 µl of saline. An imals in the control group (n=5) were subjected to intrathecal administration of 2×10^6 UCB-MC transduced with Ad5 carrying reporter green fluorescent protein gene. Three days after ex vivo gene delivery brain ischemia was induced in animals from both groups by permanent occlusion of the middle cerebral artery (MCA). Three weeks after the surgery histological study of rat brain revealed a local infarct in parietal cortex. Morphometric analysis demonstrated that in rats receiving preventive ex vivo gene therapy the infarct area was significantly smaller 2.47 (1.39, 4.51) mm^3 in comparison with the control animals 17.12 (7.26, 72.17) mm^3($p<0.05$). These results demonstrate that intrathecal injection of genetically engineered UCB-MC over-expressing therapeutic molecules (VEGF, GDNF and NCAM) at risk of a stroke may enhance the survivability of neural cells with the onset of a stroke. This study was supported by the grant of Russian Science Foundation No 19-75-10030.

P2218/B468

Pyridoxine Deficiency Affects to Expression of V-type Proton Atpase and Guanine Nucleotide-binding Protein in the Hippocampus.

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In the previous studies, pyridoxine supplementation significantly increased the hippocampal functions by upregulating phosphorylation of CREB signaling. In the present study, we investigated the effects of pyridoxine deficiency on hippocampal neurogenesis and proteins based on immunohistochemistry and proteomic approach, respectively. Eight-week-old mice received control diet (7 mg/kg) or pyridoxine deficient diet (4.4 ug/kg) for 4 weeks and sacrifice the animals for immunohistochemistry and 2D gel electrophoresis and subsequently for matrix-assisted laser desorption/ionization. Mice fed with pyridoxine deficient diet significantly decreased the cell proliferation and differentiated neuroblasts in the dentate gyrus based on Ki67 and doublecortin immunohistochemistry. In the proteomic study, among 378 paired proteins, 16 proteins were identified as significantly changed (2 folds or more) proteins. Especially, V-type proton ATPase and guanine nucleotide-binding protein was prominently decreased in pyridoxine deficient diet treated groups compared to that in the control group. These results suggest that pyridoxine has an essential role for hippocampal neurogenesis by modulating V-type proton ATPase and guanine nucleotide-binding protein in the hippocampus.

P2219/B469

Analysis of the expression profile of mRNAs in brain of hyperglycemic rats.

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Diabetes mellitus (DM) is a metabolic disease characterized by a failure in the secretion of insulin or by the ineffectiveness of this hormone on its target tissues, which leads to the development of hyperglycemia. This condition is harmful to many tissues, which leads to the development of comorbidities such as neuropathies, retinopathies and nephropathies, which in turn have been related to changes in the vasculature and systemic blood pressure, however it is unclear whether hyperglycemia promotes the appearance of these changes. Using the microarray technique, we analyzed the expression of ~ 5000 genes from the messenger ribonucleic acid (mRNA) of brain from normal and hyperglycemic rats (*Rattus Norvergicus*) (>250 mg / dL). Thus, we found that sustained hyperglycemia (72h) modifies the expression of genes encoding proteins important for the regulation of vascular function. Although we found a total of 18 positively regulated genes, only 3 of these genes encode proteins important for vascular function. 1) arginine succinate synthase (ASS), an enzyme involved in the synthesis of citrulline and nitric oxide. 2) Dysadherin (FXYD5), a transmembrane protein that positively regulates the activity of Na + -K + -ATPase and E-cadherin. The overexpression of this gene could be a mechanism of vascular protection, since it has been reported that the expression of Dysadherin is suppressed in spontaneously hypertensive rats. An d 3) the beta subunit of the epithelial sodium channel, which is expressed in hypothalamic neurons that secrete vasopressin, a potent vasoconstrictor

hormone that plays an essential role in water balance and regulation of blood pressure. None of the 26 genes negatively regulated seems to be related to the control of vascular function.

P2220/B470

Molecular Characterization of the Parkinsonian Brain After Long-term Stem Cell Transplantation.

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To improve Parkinson's disease (PD) belonging to neurodegenerative disease, stem cell therapy is known to functionally enhance nigrostriatal dopaminergic neurons. Despite these functional recoveries, the long-term state of stem cells in vivo and their relationship with surrounding cells have not been clearly studied. In previous study, we examined a three dimensional structure of intact brain tissue after bone marrow mesenchymal stem cell (MSC) treatment at short-term induction in PD. In this study, we investigated in vivo proof-of-concept and their microenvironment of MSCs 1-year after transplantation into 6-hydroxydopamine hydrobromide-induced rat PD model at the medial forebrain. One year after surgery, amphetamine-induced rotation test was performed to evaluate the degree of the dopaminergic lesion. For transplantation, MSCs were injected into the femoral vein. One day after the final behavior test, the rats were anaesthetized, and perfused with a mixture of 4% paraformaldehyde, 4% acrylamaide, 0.05% bis-acrylamide, 0.25 VA044 in PBS. Isolated brains were incubated in 4°C for 2 days, and then temperature was increased to 37°C to induce polymerization for 2.5 hours. Hydrogel-embedded brains were plated in 10~60V organ-electrophoresis system, circulating 200 mM sodium borate buffer with 4% SDS. For three dimensional interface of graft-host axis, it was visualized by immunofluorescence to MSC-positive cells (PKH26-labelled), polysialylated neural cell adhesion molecule, and/or neuron-specific class III beta-tubulin or NeuN marker. At this site, we analyzed RNA-Sequencing to confirm the change of gene expression between PD and PD transplanted with MSC group. Using Excel-based Differentially Expressed Gene an alysis tool, we analyzed the categories related to cell migration and neurogenesis between the two groups. Furthermore, GO & Pathway and Network an alysis was mainly performed on the specifically changed genes. Taken together, our results demonstrate distinct pathophysiological and molecular patterns of in vivo responses between PD and PD transplanted with MSCs suggest structural and molecular-based differential features as candidate tool for the evaluation of stem cell during long-term therapy. This study was funded by NRF-2019R1H1A1035608.

P2221/B471

Cuprizone Affects Hypothermia-induced Neuroprotection and Enhanced Neuroblast Differentiation in the Gerbil Hippocampus After Ischemia.

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Cuprizone causes demyelination in the pyramidal cells of the hippocampal CA1 region and activates the microglia and astrocytes in the hippocampus. In contrast, hypothermia protects neurons from ischemic damage in the gerbil hippocampus. Herein, we investigated the effects of cuprizone on neuronal death and activation of astrocytes and microglia in the normothermic and hypothermic hippocampus of gerbils. Cuprizone was supplemented by food at 0.2% *ad libitum* for six weeks; thereafter, gerbils received transient forebrain ischemia with or without hypothermic preconditioning. Two weeks after ischemic surgery, animals were sacrificed, and cresyl violet staining and immunohistochemistry by glial

fibrillary acidic protein (GFAP), ionized calcium-binding adapter molecule 1 (Iba-1), Ki67 and doublecortin (DCX) were conducted to reveal the effect of cuprizone on brain ischemia. Brain ischemia decreased cell survival (cresyl violet staining) and increased reactive microglia (Iba-1-positive), and astrocytes (GFAP-positive) in the hippocampal CA1 region. Ischemia also increased proliferating (Ki67-positive) cells and differentiating (DCX-positive) neuroblasts in the dentate gyrus. In contrast, hypothermic conditioning ameliorated these changes in the gerbil hippocampal CA1 region and dentate gyrus. However, cuprizone treatment decreased cell survival induced by hypothermic preconditioning and increased reactive microglia and astrocytes in the hippocampal CA1 region. Cuprizone also decreased the ischemia-induced increases in proliferating cells and differentiated neuroblasts in the dentate gyrus. These changes occur because the protective effect of hypothermia in ischemic damage is disrupted by cuprizone administration. In addition, cuprizone decreases cell proliferation and neuroblast differentiation in the dentate gyrus, induced by transient forebrain ischemia. Myelinated nerve fibers in the hippocampus may be associated with cell survival in the gerbil hippocampal CA1 region and neuroblast differentiation, induced by hypothermic therapy, during transient forebrain ischemia.

P2222/B472

Chaperonin Containing Tcp-1 (cct) Complex Negatively Regulates Tau Accumulation and Toxicity in a Drosophila Model of Tau Toxicity.

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Abnormal accumulation of microtubule-associated protein tau is associated with a number of neurodegenerative diseases including Alzheimer's disease. In disease brains, tau is hyperphosphorylated and misfolded, and accumulation of those tau species is believed to cause neuronal death. Chaperonin containing TCP-1 (CCT) is a complex that assists folding of the proteins, including cytoskeletal components, and has been reported to control protein aggregation and degradation. However, the role of chaperones in tau toxicity is not fully understood. Here we show that CCT negatively regulates tau accumulation and toxicity in a Drosophila model of tau toxicity. In this model, expression of human tau in the fly retina causes neurodegenerationl. CCT is a large double-ring complex composed of eight paralogous subunits (CCT1-CCT8). We found that RNAi-mediated knockdown of CCT1 reduces tau levels. CCT1 knockdown did not affect mRNA levels of tau, suggesting that CCT regulates the turnover of tau protein. We also found that overexpression of CCT2 increased tau levels and enhanced tau-induced neurodegeneration. Next, we investigated if CCT specifically affects pathological tau species. Tau is highly phosphorylated in the disease brains, and among the disease-associated phosphorylation sites, its phosphorylation at Ser262/356 is reported to promote tau accumulation and enhance its toxicity. We tested whether tau phosphorylation at these sites affects the effect of CCT on tau levels by using transgenic flies expressing tau carrying alanine substitutions at Ser262/356. We found that CCT1 knockdown does not reduce the levels of tau, suggesting that CCT interacts with tau species phosphorylated at these sites. Our results suggest that CCT can enhance the accumulation of abnormal tau species. Since dysregulation of molecular chaperone network has been suggested in brain tissues from human aging and neurodegenerative diseases, further studies of the mechanisms by which CCT regulates tau levels will enhance our understanding of disease pathogenesis.

P2223/B473

Identification of *Poe* as a Novel Modifier of Par-1 Activity in *Drosophila* and Its Role in Neurodegeneration in an Alzheimer'S Disease Model.

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partition-defective kinase 1 (Par-1) is an evolutionary conserved serine-threonine protein kinase and play critical roles in cell polarity, neuronal differentiation, and microtubule stability. Mammalian homologs of Par-1, microtubule affinity regulating kinase (MARK) 1-4, have been linked to neurological diseases. MARKs phosphorylate microtubule-associated protein tau, which is hyperphosphorylated and accumulated in neurodegenerative diseases, including Alzheimer's disease. Elevated MARK activity is found with tau lesions in Alzheimer's disease brains, and a mutation in MARK4 increases the risk of Alzheimer's disease. Elevated levels of MARK1 have been linked to autism spectrum disorder. These reports suggest that disruption of MARK/Par-1 contribute to the pathogenesis of neurodegenerative diseases. However, the regulatory mechanisms of MARK/Par-1 activity in neurons are not fully understood. Here we report that *purity of essence* (*poe*) is a negative regulator of Par-1. From a genetic screen, we identified *poe* as a modifier of Par-1 protein levels in the fly retina. Western blotting revealed that RNAi-mediated knockdown of *poe* reduces the protein levels of Par-1. mRNA levels of Par-1 was not affected, suggesting that *poe* affects the turnover of Par-1 protein. *poe* knockdown did not reduce firefly luciferase expressed in the retina, indicating that the reduction in Par-1 by *poe* knckdown is not due to the enhancement of general protein degradation. We also found that *poe* overexpression increased Par-1 protein levels. To test *poe* affects Par-1 activity, we used a fly model of human tau toxicity. In this model, human tau is overexpressed in the eye, and it is phosphorylation at Ser262 by endogenous Par-1. We found that *poe* knockdown reduces tau phosphorylation at Ser262, indicating that *poe* negatively regulates Par-1 activity. *poe* is suggested to act as an E3 ubiquitin-protein ligase. Par-1 is ubiquitinated and degraded by Skp, Cullin, F-box containing complex (SCF), and this process is triggered by phosphorylation of Par-1 at Thr408. To ask whether *poe* is involved in this process, we tested the effect of *poe* knockdown on a mutant form of Par-1 with substitution of Thr408 to unphosphorylatable Alanine (Par-1^{T408A}). *poe* knockdown significantly reduced the levels of Par-1^{T408A}, suggesting that poe regulates Par-1 metabolism in a different mechanism. In summary, our results indicate that *poe* negatively regulates the degradation of Par-1 protein. Further studies will reveal a novel mechanism that regulates Par-1/MARK activity and may shed light on its dysregulation in disease pathogenesis.

P2224/B474

New Directions in Neurodegenerative Disease: Delineating the Histone Modification Landscape of ALS.

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Amyotrophic lateral sclerosis (ALS) is a fatal and incurable neurodegenerative disease that affects cells in the brain and the spinal cord. ALS has been linked to mutations in many genes including SOD1, FUS, TDP- 43 and C9orf72. Interestingly, the protein products of these genes accumulate in inclusions within affected neurons. Eukaryotic DNA is packaged into chromatin, a highly organized protein-DNA complex. Changes in the structure of chromatin are sufficient to cause heritable phenotypic changes termed epigenetic. Epigenetic mechanisms include the methylation of DNA and the covalent post-translational

modification of histone proteins. Up until now, the majority of investigations into ALS epigenetics have focused on DNA methylation. Comprehensive characterization of the histone modification landscape in ALS is still lacking. We hypothesize that post-translational modification (PTM) of histones plays a role in the cellular demise observed in ALS. We have discovered distinct histone modification profiles associated with FUS, TDP-43 and C9orf72 proteinopathies in yeast and human models. Remarkably, we discovered distinct changes in histone modification profiles for each proteinopathy model evidencing that histone PTM changes are not associated with general protein aggregation toxicity pathways. Furthermore, we have identified small molecules that diminish the toxicity of FUS overexpression. Our data corroborates previous findings in different model systems, while significantly broadening our knowledge of the epigenetic landscape of neurodegenerative disease. Our findings highlight novel epigenetic mechanisms at play in ALS. Epigenetic processes are highly accessible targets for pharmaceutical treatments and thus they can lead to novel, alternative approaches in the treatment of ALS and other neurodegenerative diseases.

P2225/B475

Cortical Synaptic and Mitochondrial Dysfunction in Mouse Models of Huntington'S Disease.

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Cortical synaptic and mitochondrial dysfunction in mouse models of Huntington's disease

Huntington's disease (HD) is a fatal movement disorder. Due to the CAG repeats expansion (>36) of exon 1 in the Huntingtin (HTT) gene, HD is likely resulted from accumulation of mutant HTT (mHTT) protein with increased polyglutamine (polyQ) tract near the N terminus. Despite of intensive research, the underlying mechanisms of mHTT eliciting HD is still not clear. We used two related HD mouse models to explore the cellular mechanisms of the pathogenesis in HD. One is the BACHD mouse that expresses a moderate level of the full-length human mHTT transgene (polyQ97) using a bacterial artificial chromosome (BAC). The second model is based on BACHD except the N-terminal 17-residues were removed (ΔN17-BACHD). We first examined the formation of synapses by IF using anti-synapsin I for pre-synapses and anti-post synaptic density 95 (PSD95) for post-synapses. Our findings are: a) the synaptic formation began to form at DIV14; b) No difference between WT and BACHD cortical neurons was observed at DIV14; c) Starting at DIV21, BACHD neurons showed a progressive reduction in synapses up till DIV35. By contrast, the synapses in ΔN17 BACHD cortical neurons were significantly increased at DIV28 and DIV35. We then used JC-9 to measure mitochondrial activity. Our results demonstrated: a) BACHD primary cortical neurons showed accumulation of multiple mitochondria accumulated locally and formed inclusion-body like structures in the processes at DIV8. Mitochondrial membrane potentials were also significantly reduced at DIV13 in these neurons. Again, the data for ΔN17 BACHD cortical neurons were contrary to BACHD i.e an increase in mitochondrial membrane potential. We thus conclude that both of BACHD and ΔN17 BACHD neurons showed abnormal synaptic formation and mitochondrial activity, albeit the two models displayed opposite effects. Understanding the molecular and cellular mechanisms underlying the difference between the two HD models will yield important insights into HD pathogenesis.

P2226/B476

Effects of Distressed SH-SY5Y Cells on the Migration and Aggregation of Activated Bv2 Cells.

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Parkinson's disease (PD) is a debilitating disorder that affects dopaminergic neurons of the Substantia Nigra. The degeneration of dopaminergic neurons begins decades before symptoms are first seen. One possible contributor to PD may be systemic activation of inflammation or over-activation of microglia in the brain. While microglia are necessary for brain health, there is evidence that over-activated microglia, as seen in the LRRK2 mutation associated with PD, can lead to increased susceptibility of dopaminergic neurons to injury. We hypothesize that activated microglia can be pushed from a physiological to pathological state in the presence of distressed neurons. Therefore, we developed an in *vitro* model of PD where we co-culture distressed SH-SY5Y cells with lipopolysaccharide (LPS)-activated BV2 cells. We used bright-field and fluorescence microscopy to quantitate the effects of distressed SH-SY5Y cells on BV2 migration and aggregation. In our model, increased LPS concentrations had a direct relationship with increase microglia aggregation and migration. These effects were not evident when activated BV2 cells were co-cultured with healthy SH-SY5Y cells. Ongoing studies are investigating the effects of other pro-inflammatory factors on BV2 activation states.

P2227/B477

2-carba Cyclic Phosphatidic Acid Contributes to the Neuroprotection in Stab-wounded Cerebral Cortex *Via* Regulation of Astrocytes.

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Traumatic brain injury (TBI) is caused by physical damage, and the brain structure is disrupted due to the biomechanical insult to the cranium. We previously revealed that 2-carba cyclic phosphatidic acid (2ccPA) significantly suppresses the hemorrhage level and inflammation *via* the regulation of microglial activation in the stab-wounded cerebral cortex as a TBI mouse model. 2ccPA is a metabolically stabilized derivative of cyclic phosphatidic acid (cPA), and with phosphate oxygen is replaced with a methylene group at the *sn*-2 position of cPA. Furthermore, we have already reported that the suppression of hemorrhage level by 2ccPA is related to the recovering from blood-brain barrier (BBB) breakdown caused by the TBI. Astrocytes, one of the major components of BBB, take a critical role in neuroprotection. However, the mechanism how astrocytes are involved in the promotion of the neuroprotection by 2ccPA in the stab-wounded cortex remains unknown. In addition, it is known that the expression of tenascin-C (TN-C), one of extracellular matrix proteins, is upregulated in the reactive astrocytes after experimental subarachnoid hemorrhage and suggests that delay of the recovery from BBB breakdown. In this study, to investigate the effect of 2ccPA on the neuroprotective function through astrocytes after the brain injury, we analyzed the glial fibrillary acidic protein (GFAP), an astrocyte marker and upregulated when it became reactivated and TN-C, expression levels and neuronal survival in the stab-wounded mouse cortex. We found that the administration of 2ccPA to brain injured mice decreased the *Gfap* mRNA and protein expression levels. Not only that, 2ccPA reduced the number of GFAP- and Sox9 (an activated astrocyte marker)- double positive astrocytes, suggesting that 2ccPA attenuates the activation of astrocytes after the stab wound. Moreover, to analyze the effect of 2ccPA on the neuro survival in the wounded regions. We revealed that 2ccPA reduced the number of the activated caspase-3 positive cells, indicating that 2ccPA suppresses the neuronal cell death. Therefore,

these results suggest that 2ccPA is involved in neuroprotection *via* the regulation of astrocyte activity in the stab-wounded cortex.

P2228/B478

Secretion of Tau by Vamp8: a Novel Pathway for Tau Clearance.

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In Alzheimer's disease (AD), tau, a neuronal microtubule-associated protein becomes hyperphosphorylated, aggregates and accumulates in the somato-dendritic compartment. In parallel to its intracellular accumulation, tau was also shown to accumulate in the extracellular space in AD. Indeed, tau was detected in the cerebrospinal fluid during the progression of the disease, which was believed to correlate with neuronal cell death. However, recent studies including ours have reported that neurons can secrete tau. Based on the fact that both intracellular and extracellular accumulations of tau were shown to be detrimental to neurons, we have investigated tau secretory pathway. Tau secretion occurs through unconventional secretory pathways, which were known to involve structures such as late endosomes, autophagosomes and lysosomes, membranous organelles that can fuse with the plasma membrane to release their content. Previously, we reported that Rab7A associated with late endosomes was implicated in tau secretion. In this study, we explored whether vesicle-associated membrane protein 8 (VAMP8), a SNARE involved in endosomal fusion and exocytosis of several secretory cells was involved in tau secretion. The effects of VAMP8 on tau secretion were examined in the neuroblastoma cell line N2A using immunoblot, dot blot and TIRF microscopy. The overexpression of VAMP8 significantly increased tau secretion resulting in a decrease of its intracellular accumulation in N2A. The same effects of VAMP8 overexpression was observed with tau mutants linked to Fronto-Temporal Dementia with Parkinsonism, P301L and R406W, and with alpha-synuclein, indicating that VAMP8 could be used for clearance of multiple proteins involved in neurodegenerative diseases. Using TIRF microscopy, it was possible to observe the fusion of VAMP8-positive vesicles with the plasma membrane, which was associated with the depletion of tau signal in the cytoplasm. We previously demonstrated that cleavage of tau by caspase-3 increased its secretion. This was also observed when tau secretion was increased by VAMP8 overexpression. Extracellular tau was shown to be responsible for the propagation of tau pathology in the brain. In this context, extracellular tau is presently a therapeutic target in several clinical trials where it is neutralized by an anti-tau antibody. From the above observations, one can speculate that the increase of tau secretion combined with the capture of extracellular tau by an antibody could be an efficient approach to prevent tau intracellular accumulation as well as the propagation of tau pathology in AD brain.

P2229/B479

The Role of Lamin a and Progerin in Brain Aging and Neurodegenerative Diseases.

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Neurodegenerative diseases, which are featured by progressive neuronal impairment, are one of the most severe public health problems over the world. Alzheimer's disease (AD) is the most common neurodegenerative disease, characterized by amyloid plaques and tau fibrils. The nuclear lamina, consisting of a and B type lamins, plays a structural supporting role for most cell types including brain cells, and it is associated with brain development and neuronal survival. While B type lamins are universally expressed in brain cells, the expression of a type lamins in the brain is generally repressed.

Interestingly, a recent study showed an up-regulation of a type lamins in the hippocampus in the late-stage AD. Here, we aim to study the potential role of a type lamins and a lamin a mutant form, progerin in brain aging and neurodegenerative diseases. Using a neural progenitor cell (NPC) line which can be differentiated into neurons, we first examined the regulation of type a and B lamin expression during differentiation. We found differential regulations, at transcriptional and translational levels, of lamins A, B and C. We also used lentiviral constructs to overexpress lamin a and progerin in NPCs and evaluated their impacts on neuronal function. Our results suggest that overexpression of lamin a and progerin could induce nuclear blebbing, increase apoptosis events and cell cycle re-entry. We further postulate that induced lamin a and progerin with age may contribute to neurodegeneration.

P2230/B480

***Drosophila* Models of ALS Identified the Context Impact on the Toxicity of Expanded GGGGCC Repeats.**

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An intronic hexanucleotide repeat expansion in *C9ORF72* causes familial and sporadic Amyotrophic Lateral Sclerosis (ALS) and frontotemporal dementia (FTD). This repeat is thought to elicit toxicity through RNA mediated protein sequestration and repeat-associated non-ATG (RAN) translation. We generated transgenic flies expressing an artificial intron containing interrupted GGGGCC repeats within a GFP reporter. 484 intronic repeats elicited minimal alterations in eye morphology, viability, longevity, or larval crawling but did trigger RNA foci formation. In contrast, insertion of 28 GGGGCC repeats into the 5' leader upstream of GFP was toxic in some reading frames. To investigate whether the GGGGCC repeat toxicity is also modified by the surrounding context of human *C9ORF72* gene, phenotype analyses were performed on several transgenic fly lines either expressing expanded GGGGCC repeat alone or expressing the GGGGCC repeats along with the 3' downstream sequence encoding hypothetical C-terminal fragments. It was found that the 3' downstream sequence in these expanded repeats partially attenuated the repeat toxicity. Similar findings of the 3'-downstream sequence attenuations was also confirmed in cell culture transfected with similar constructs. In summary, the toxicity of expanded GGGGCC repeats in *Drosophila* ALS models is highly dependent on the context: while intronic expanded repeats are largely nontoxic in *Drosophila*, exonic repeat toxicity is further modified by the intrinsic 3' downstream sequence. These findings are meaningful for better understanding C9ALS disease pathogenesis.

P2231/B481

Phenotypic Screening and Functional Genetics in Ftd/als.

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Frontotemporal Dementia (FTD) and Amyotrophic Lateral Sclerosis (ALS) are adult-onset neurodegenerative diseases that are closely related clinically, pathologically, and genetically. Therefore, it is likely that converging disease pathways exist and can be used both to enhance our understanding of basic FTD/ALS biology and as leads for therapeutic intervention. FTD/ALS is often inherited and causal mutations are highly penetrant, indicating that these mutations have large effects on critical cellular

pathways. Approximately half of the genes known to cause FTD/ALS encode proteins that regulate endolysosomal biology or play a crucial role in regulating proteostasis. Our own unpublished data implicates lysosomal dysfunction as a central pathophysiological consequence of mutations in *GRN*due to defects in endolysosomal acidification and/or maturation, building upon previously established work from my mentor demonstrating that lysosomes are a key player in FTD/ALS-related neurodegeneration. Here, we propose to use our modified i^{3}Neuron platform to facilitate systematic knockdown of large panels of FTD/ALS-associated genes through CRISPR interference (CRISPRi) to interrogate Gene-Drug and Gene-Gene interactions. From this, we will generate genetic interaction maps of pairs of FTD/ALS genes in iPSC-derived neurons, identifying common pathogenic changes elicited by disease genes related to several key cellular processes, including endolysosomal acidification and maturation, lysosomal ion homeostasis, selective autophagy, organelle quality control, and neuronal survival. We will also use drug libraries with well-characterized molecular targets in combination with CRISPRi knockdowns to more precisely define sensitized pathways in FTD/ALS. Through these efforts, we hope to lay the groundwork for improved molecular understanding and therapeutic discovery.

P2232/B482

Identification of Cell Receptors Involved in the Propagation of Tau Aggregates.

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Prion-like propagation of aberrant protein aggregates in the brains of neurodegenerative disease (ND) patients involves the release of misfolded proteins from neurons, their uptake by nearby cells, and induction of endogenous protein aggregation and proteotoxic stress in recipient cells. Inflammation response is another insult that has been linked to the interaction of misfolded proteins with cells in ND. Little is known about how misfolded proteins either in a monomeric state or as aggregates interact with the plasma membrane and the resulting pathophysiological impact. To understand how misfolded proteins interact with the plasma membrane to initiate prion-like propagation and potential signaling pathway, we used Tau as a model because it is misfolding prone protein and the transmission of Tau aggregates between cells impacts the progression of Alzheimer's disease. We employed spatially-restricted proteomic mapping using APEX2-tagged Tau. APEX2 is an engineered peroxidase that can covalently tag proximal proteins with a biotin molecule in the presence of biotin-phenoxyl radicals. Following the binding of Tau-APEX2 to the cell surface, biotinylation labeling was initiated by addition of biotin-phenoxyl, and biotinylated plasma membrane proteins were then enriched by streptavidin-affinity purification and identified by mass spectrometry. Using this approach, we identified a member of the integrin family as the potential Tau binding receptor. Integrins are a major cell adhesion molecule that binds extracellular matrix proteins and are involved in maintenance of neuronal functions. Using in-vitro and cell-based pull-down assay, we confirmed the interaction between integrins and both monomeric and oligomerized Tau. We also found that over-expression of integrin family members in 293T cells can increase Tau binding, and conversely, knockout of endogenous integrins in SH-SY5Y cells by CRISPR/Cas9 decreases Tau-plasma membrane interaction. In order to reveal the physiological consequence of Tau-plasma membrane interaction, RNAseq was employed to identify gene expression differences between primary astrocyte treated with Tau monomers or fibrils. Partial overlapping but distinct sets of induced genes were identified. Typically, we found that cytokine and chemokine induced neuro-inflammation was significantly increased in Tau-fibril treated astrocytes, which also upregulate reactive astrocyte marker genes, compared to those treated with Tau monomers. Interestingly, knockdown of integrin can significantly reduce the activation of inflammation. In summary, we have

identified a receptor for misfolded Tau, which not only mediates the uptake and propagation of Tau fibrils, but also regulates the inflammation in response to misfolded proteins.

P2233/B483

Bait RNA Oligonucleotides to an tagonize Pathological Protein Aggregation in ALS/FTD.

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Aberrant aggregation of RNA-binding proteins, such as TDP-43 and FUS, is a common neuropathological hallmark of neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Current cellular models of these neurodegenerative proteinopathies often rely on the overexpression of disease-linked mutant proteins to induce pathological protein aggregation. However, mutations in the TARDBP and FUS genes each only account for ~5% of familial ALS (fALS) cases. The vast majority of patients do not harbor mutations in these genes, yet still experience aggregation of these proteins. Similarly, rodent models of ALS produced from the overexpression of these mutant proteins have been historically unreliable and often fail to produce pathologically-relevant inclusions. Here we present a novel optogenetic-based technique to induce pathological aggregation of TDP-43 and FUS with a previously unachievable level of spatial and temporal control (optoTDP43/optoFUS). Using this approach, we discovered that the RNA-binding status of TDP-43 and FUS dictate their propensity to undergo liquid-liquid phase separation (LLPS) and form pathological inclusions. We also show that these aberrant intracellular phase transitions are toxic to human neuronal cultures and that bait RNA oligonucleotides (bONs) mimicking known TDP-43 or FUS binding sequences can prevent light-induced phase transitions and rescue resulting neurotoxicity. In order to work towards a more therapeutically-relevant paradigm, we also investigate the ability of bONs to disaggregate existing TDP-43 and FUS inclusions and examine resulting effects on neuronal survival. This optogenetic strategy can be further applied to a number of different disorders and will aid in the identification of negative regulators of pathological protein aggregation, as well as in-depth investigations into the effects of these pathological aggregates on various cellular pathways and downstream pathological processes.

P2234/B484

Myosin-7B Facilitates Heparan Sulfate Proteoglycan-mediated Endocytosis of α-Synuclein Fibrils**.**

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Cell-to-cell transmission of misfolding-prone α-synuclein (α-Syn) has emerged as a key pathological event in Parkinson's disease. This process comprises two poorly characterized cellular pathways: release of α-Syn by unconventional protein secretion and uptake of α-Syn-bearing fibrils via clathrin-mediated endocytosis (CME). Using a CRISPR-mediated knockout screen, we identify SLC35B2 and Myosin-7B (MYO7B) as critical regulators for endocytosis of α-Syn preformed fibrils (α-Syn PFF). SLC35B2 is a membrane transporter involved in the synthesis of cell surface heparan sulfate proteoglycan (HSPG), whose sugar moieties bearing linearized negative charges can insert into a groove formed by two K-X-K motifs clustered in α-Syn PFF. MYO7B, an unconventional myosin acts with actin filaments to maintain membrane dynamics at plasma membrane domains enriched in clathrin. Without MYO7B, a fraction of clathrin-coated pits fail to be severed from the plasma membrane, causing accumulation of large clathrin-containing scars on the cell surface. Intriguingly, the functions of MYO7B and SLC35B2 are only

restricted to the uptake of α-Syn PFF and other polycation-bearing cargos that enter cells via HSPG. We propose that the maturation and optimal function of clathrin-coated pits (CCP) in HSPG-dependent endocytosis requires additional force generated by MYO7B and the actin network.

P2235/B485
A Robust Double Mutant Mouse Model of Hereditary Spastic Paraplegia Reveals Expanded Endoplasmic Reticulum in Axons.
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Hereditary spastic paraplegias (HSPs) are neurologic disorders affecting the longest corticospinal axons, resulting in lower extremity spasticity. The most common autosomal dominant forms are caused by mutations in genes that encode spastin (SPG4), atlastin-1 (SPG3A) and REEP1 (SPG31). These proteins harbor hydrophobic hairpin domains, bind one another, and play an important role in shaping the tubular ER network. To date, most studies on atlastin-1 and REEP1 have been performed in cultured cells, although they are highly enriched in the central nervous system. Here, we report a robust double mutant mouse model of HSP in which a GTPase-defective atlastin-1 has been generated with a K80A missense change and the *Reep1* gene has been disrupted. These double mutant mice show significant behavioral changes in hindlimb function tests. The hindlimb weakness appears as early as 4 weeks and continues to progress, with euthanasia typically performed around 6-8 months due to severe motor impairment. Interestingly, ER within corticospinal tract axons in spinal cord is expanded dramatically in the double mutant mice as compared to wild-type and single mutant mice. This robust mouse model will prove useful for studying the roles of impairments in atlastin-1 and REEP1 function that result in ER morphology changes that underlie the most common forms of HSP, and it also may help to identify potential molecular targets for the development of therapeutics.

Neuronal Morphogenesis and Axo-Dendritic Organization

P2236/B486
A Pair of E3 Ubiquitin Ligases Compete to Regulate Filopodial Dynamics and Axon Guidance.
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Appropriate axon guidance is necessary to form accurate neuronal connections. The cytoskeletal rich growth cone at the tip of an extending axon senses and responds to extracellular guidance cues in order to navigate axon growth toward appropriate targets. Filopodia at the periphery of the growth cone have long been considered sensors for axon guidance cues, yet how they perceive and respond to extracellular cues remains ill-defined. Our work has found that in embryonic murine cortical neurons, the filopodial actin polymerase VASP is regulated via TRIM9-dependent non-degradative ubiquitination, and that appropriate VASP ubiquitination and deubiquitination are required for axon turning in response to the guidance cue netrin-1. Here we show that the TRIM9-related E3 ubiquitin ligase TRIM67 antagonizes TRIM9-dependent VASP ubiquitination. Ubiquitination alters the dynamics of VASP at filopodia tips and the stability of growth cone filopodia. TRIM67 ligase activity is surprisingly required for antagonizing VASP ubiquitination, as well as its strong interaction with VASP, which outcomes TRIM9. By antagonizing VASP ubiquitination, TRIM67 increases filopodia stability and promotes responses to netrin. This antagonistic role in VASP ubiquitination is required for netrin-1 dependent filopodial and

growth cone responses and netrin-dependent axon branching. Using a compartmentalized axon guidance microfluidic device, which exposes only axons to a stable gradient of netrin, we find that TRIM67 is required for netrin-dependent axon turning responses in vitro. In vivo, netrin is required for the formation of the corpus callosum, a major midline axon projection in the cortex. We find that deletion of *Trim67* delays the formation of this fiber tract, and the resultant commissure is thinner than in wildtype littermates. This phenotype is the opposite phenotype of mice in which *Trim9* is deleted. We suggest a novel yin yang model: coordinated regulation of non-degradative VASP ubiquitination by a pair of ligases is a critical element of netrin-dependent filopodial stability, growth cone response, and axon guidance.

P2237/B487

Transcriptomic an alysis of CAD Cell Differentiation.

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CAD cells are a model neuron-like cell line derived from Cath.A cells a mouse central nervous system catecholaminergic cell line. Serum-starvation of CAD cells induces the production of long neurite extensions over the course of 5 days. These cells have been used in multiple published studies of neuronal function, and in other work from our lab CAD cells are serving as the base cell type for characterization of proteins involved in mitochondrial traffic and neuronal development via stable shRNA expression. CAD cells bear a morphological resemblance to isolated neurons when observed by either light microscopy or electron microscopy. We hypothesized that they would also bear a similar pattern of gene expression changes compared to developing neurons. We approached this hypothesis by carrying out comparative transcriptomic RNAseq analysis comparing undifferentiated and differentiated wild type CAD cells. We identified approximately 1900 mRNA transcripts that changed expression levels between undifferentiated and differentiated cells (-1>logbase2>1, padj<0.1). Using immunofluorescence microscopy, we demonstrated that protein level changes for a select number of these genes mirrors the changes in mRNA levels. Using Pathview, we identified pathways as a starting point where gene expression within the pathway changed. We examined a subset of the 80 identified pathways that were related to neuronal function. We also compared the changes in gene expression observed in CAD cells to changes in gene expression observed in cortical embryonic stem cells differentiated in vitro (http://cortecon.neuralsci.org/) and were able to identify a set of genes and pathways whose expression changes in CAD cells mirror those observed in developing cortical neurons. These data provide a more detailed understanding of the neuron-like character of differentiated CAD cells.

P2238/B488

Kansl1 Haploinsufficiency Suppresses Postnatal Neurogenesis and Normal Neural Behavior in Mice.

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Koolen-de Vries syndrome is a neurodevelopmental disorder characterized by developmental delay and intellectual disability. The syndrome is caused by either mutations in the *KAT8 regulatory NSL complex complex subunit 1* (*KANSL1*) gene or microdeletions in the 17q21.31 chromosomal region that includes *KANSL1*, leading to *KANSL1* haploinsufficiency. However, the pathogenic mechanism of how *KANSL1* haploinsufficiency disrupts intellectual function is unknown. Here, we report that *Kansl1*

haploinsufficient mice show learning and memory impairments, which recapitulates intellectual dysfunction in human Koolen-de Vries syndrome. The Morris water maze test revealed that *Kansl1* haploinsufficient mice spent more time in finding the platform than wild type controls. The novel object test confirmed the disrupted intellectual function of identifying novel object in *Kansl1* haploinsufficient mice. The social novelty test using the three-chamber paradigm also showed abnormal recognition in the mutant mice. Interestingly, *Kansl1* haploinsufficient mice exhibited the reduced size of the olfactory bulb, indicating disrupted neurogenesis and migration of subventricular zone neural progenitors. Additionally, neural progenitor proliferation in another neurogenic niche, the hippocampal dentate gyrus, was decreased in *Kansl1* haploinsufficient mice. The number of proliferative progenitors identified by short-pulse BrdU tracing and phospho-histone H3 (Ser 10) immunostaining was reduced in the mutant mice, compared to control mice. These results demonstrate that both copies of *Kansl1* is required for postnatal neurogenesis. Combined with behavioral phenotypes of *Kansl1* haploinsufficient mice, our data suggest that impaired genesis and integration of newborn neurons in neurogenic niches may contribute to the development of intellectual dysfunction in Koolen-de Vries syndrome.

P2239/B489

Motor Neuron Morphogenesis Is Controlled by Transcriptional Activation of Phospholipid Signaling to the Actin Cytoskeleton.

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Neurons display the greatest morphological diversity among all cell types in the adult organism but the developmental pathways controlling the morphogenesis of their axons, dendrites and cell bodies remain ill understood. The objective of this study was to analyze the molecular programs controlling the morphogenesis of motor neurons innervating skeletal muscle. To this end, we analyzed the morphogenesis of two subsets of spinal motor neuron both in *vivo in* developing mouse embryos - using light sheet and dive-in microscopy - and *ex vivo* - using novel techniques of motor neuron FACS and flow cytometry (Schaller et al PNAS 2017), patch clamp recording, gene expression profiling and genetic modification. Our results show that motor neurons innervating hindlimb muscles exhibit higher rates of axon and cell body growth than motor neurons innervating axial muscles. We demonstrate that these morphogenic differences are cell-intrinsic (cell autonomous) and associated with the differential regulation of a phospholipid signaling pathway leading from activation of SGMS2, DGKb, CDS1, PI5P-4K and PLCE to actin polymerization. DGKb (diacylglycerol kinase beta) is identified as the key enzyme in this pathway since its gene knockdown or over expression is sufficient to switch the morphogenic path of hindlimb and axial motor neurons. The transcription factors FoxP1 and Hb9 act as upstream regulators of this pathway since their cross repressive actions mimic the effects of DGKb on actin polymerization as well as axon and cell body growth. In conclusion, these data unravel how transcriptional codes fine tune the morphogenesis of motor neurons through differential phospholipid signaling to the actin cytoskeleton.

P2240/B490

Snap47 and Trim67 Alter Mode of Vamp2-mediated Exocytic Fusion in Developing Cortical Neurons.

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Exocytosis is required in developing neurons to supply material for plasma membrane expansion during morphogenesis. Classically, two modes of fusion have been reported. During full-vesicle-fusion (FVF), the fusion pore dilates and the vesicle collapses into the membrane. During kiss-and-run fusion (KNR) the fusion pore opens transiently for secretion, without adding plasma membrane material. We hypothesized that during neuronal morphogenesis, FVF-like exocytosis predominates to supply membrane material. To test this, we exploited VAMP2-pHluorin as a fluorescent readout of fusion pore opening. Images were analyzed using our automated analysis platform. Here we introduce a novel machine-learning method using multiple classifiers, including hierarchical agglomerative clustering, dynamic time warping, and feature selection with dimensionality reduction for unsupervised categorization of exocytosis. A majority-rule committee of 28 indices selected four discrete classes. This surprising result suggests classes diverging from FVF and KNR exist. All four classes were tetanus sensitive, indicating bona fide VAMP2-mediated fusion. HEPES addition increased the half-life of fluorescence decay in two classes, indicating decay resulting from re-acidification, consistent with KNR fusion. The HEPES insensitive classes displayed diffusion of fluorescence away from the fusion site, consistent with FVF-like behavior. One KNR and one FVF class exhibited immediate fluorescence decay after fusion pore opening, whereas the others demonstrated a delay in decay onset, consistent with the pore remaining open prior to diffusion or resealing. We named the four classes instantaneous FVF (FVFi), delayed FVF (FVFd), instantaneous KNR (KNRi), and delayed KNR (KNRd). Modes were confirmed by following the fate of VAMP2 using VAMP2-TagRFP and VAMP2-pHluorin and by expressing a VAMP2 peptide that inhibits FVF. Mathematical modeling suggests FVF supplies sufficient plasma membrane material for morphogenesis in developing neurons. Deleting the E3 ubiquitin ligaseTrim67 significantly shifted the mode of exocytosis toward KNR fusion, without altering the frequency of exocytosis. We identified SNAP47 as a t-SNARE that interacts and colocalizes with TRIM67. SNAP47 levels increased upon deletion of Trim67. SNAP47 co-localized with a subset of VAMP2-mediated fusion events. Overexpression of SNAP47 in wildtype neurons partially phenocopied loss of Trim67, increasing KNRd exocytosis, whereas SNAP47 knockdown in Trim67-/- neurons rescued exocytic mode phenotypes. Together these results suggest that TRIM67 alters exocytic mode via SNAP47. This study provides novel quantitative and mechanistic insights into the modes of exocytosis and how they alter neuronal morphogenesis.

P2241/B491

Activity-dependent Neuroprotective Protein (adnp) Functions Via Multiple Distinct Mechanisms to Drive Cortical Neuronal Morphogenesis.

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Activity-dependent neuroprotective protein (Adnp) is a master regulator of ~400 genes and various signaling pathways essential to embryonic and postnatal development, and mutations in Adnp are some of the most frequent underlying Autism Spectrum Disorder and lead to a distinctive combination of clinical features. Adnp also functions in the cytoplasm during neuronal maturation to promote dendritic spine/synapse formation via microtubule interactions. Adnp's activities in earlier neuronal development

remain elusive but are important to understand how Adnp mutations result in pathology. Previous research has suggested that Adnp may play a role in promoting neurite formation. Defective neuritogenesis is currently gaining notoriety as a contributing pathogenic mechanism behind a variety of neurodevelopmental diseases, prompting us to investigate Adnp's suggested role in more detail. Our RNA-Sequencing (RNA-Seq) data suggest increased Adnp expression throughout neuritogenesis, and our cortical neuritogenesis analyses implicate a much more complex role for Adnp in neuronal morphogenesis than previously reported. Overexpression of Adnp in *vitro* leads to premature spine-like formation on all neurites, including the neurite most likely to become the axon suggesting that Adnp may function in neuronal polarization and timing of dendritic spine emergence. Knockdown of Adnp in *vitro* and in *vivo* leads to increased neurite initiation and increased elongation of the axon only. In *vivo* analysis using in *utero* electroporation revealed Adnp knockdown leads to an additional morphological deficit, disrupting the angle of the apical dendrite at P3. This may reflect altered connectivity and response to guidance cues, which is ultimately based on microtubule dynamics. Adnp is known to promote microtubule polymerization and we conclude that this defect at P3 as a result of Adnp knockdown is due to altered microtubule dynamics. *Ex vivo* time-lapse live imaging of neuritogenesis in living brain slices revealed further severe defects in neurite formation and dynamics including dilations and swellings on growing neurites from Adnp deficient cells, potentially as a result of cytoskeletal bundling and/or defective cellular transport. Further notable defects include increased neurite length but decreased growth throughout imaging, decreased growth and shrinking velocity, and increased neurite stabilization. We conclude that Adnp works via multiple distinct mechanisms to establish proper neuronal morphogenesis, which is crucial for cortical development, and that both overexpression and deficiencies in Adnp result in severe morphological defects in cortical neurons which have implications for overall cortical function.

P2242/B492

***Arid1b* Haploinsufficiency Suppresses Neurite Development and Mitochondria Function.**

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Haploinsufficiency of the *AT-rich interactive domain 1B (ARID1B)* gene, a chromatin remodeling gene, causes autism spectrum disorder and intellectual disability. Despite *ARID1B*'s critical status in neurodevelopmental pathogenesis, the consequences of *ARID1B* haploinsufficiency at the molecular and cellular level remain poorly understood. Here using *Arid1b* heterozygous knockout mice, we show that *Arid1b* haploinsufficiency suppresses outgrowth and branching of axons and dendrites in the cerebral cortex. The number and length of primary cortical dendrites were decreased in *Arid1b* haploinsufficient mice, compared with wide-type controls. Secondary, tertiary and quaternary branches as arborization indicators showed a reduction in the mutant condition. Elongation of callosal axons was also impaired in *Arid1b* haploinsufficient mice. Dendritic spines appeared abnormal as they revealed thin and short protrusions on dendrites in the mutant mice. The abnormal spines accompanied an increase in the synaptic cleft width and a decrease in the length of post-synaptic density, compared with controls mice. Intriguingly, there were reductions in the mitochondrial membrane potential and cytochrome c oxidase activity in *Arid1b* haploinsufficient neurons. Furthermore, *Arid1b* haploinsufficient neurons revealed decreased *Ntrk2* (*TrkB*) mRNA expression as well as reduced binding of acetylated histone H3 (H3K9ac) on the *Ntrk2* promoter. Together, our findings suggest that *Arid1b* haploinsufficiency leads to abnormal histone modification at K9 via energy deficiency, which is likely caused by mitochondrial dysfunction. This disrupted epigenetic status may suppress the energy-demanding process of chromatin remodeling

on the *Ntrk2* gene, thereby inhibiting *Ntrk2* expression and neurite development in *Arid1b* haploinsufficient neurons.

P2243/B493

Extracellular ATP Promotes Spiral Ganglion Neuron Maturation Via P2rx3 Receptors.

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The mammalian cochlea undergoes a highly dynamic process of growth during development. This process includes the extension of the cochlear duct along with spiral ganglion neuron (SGN) migration, followed by their maturation and synaptic contact with hair cells (HCs). In mouse, this process commences around embryonic day 12 (E12) and continues through early postnatal stages. Understanding the molecular mechanisms of precise cochlear wiring reveals principles of developmental biology and will be crucial for developing molecular-based treatments for hearing loss. Extracellular ATP is known to act as a neurotransmitter for mature neurons by activating either ionotropic or metabotropic purinergic receptors. However, little is known about their roles in development. We have found that one of the ionotropic receptor family members, P2X3, is expressed at high levels in SGNs from E12.5 to P6. We show here this developmental period includes a phase of dramatic branch pruning and hypothesized that P2X3 was involved in this process. To examine the function of P2X3 in SGN development, we examined individual SGNs from *P2x3*$^{-/-}$ cochleae after genetic labeling using *Sox2*^{CreERT2} and *R26R*^{tdTomato}. Using this approach, we found that loss of P2X*3* results in more complex branching patterns at axonal terminals and dysregulated branch refinement near SGN cell bodies around P0. These data suggest that P2X3 normally mediates a pruning process to eliminate excessive branches during early SGN development. Given that P2X3 predominantly permeates calcium and such spontaneous calcium transients coincide with the critical period of branch pruning, we further tested if P2X3 underlies spontaneous neuronal activity. Using Flou4-AM and calcium imaging, we found that a,ß,me-ATP, a P2X3 agonist, potentiates SGN calcium events and that NF110, a P2X3 antagonist, alters activity patterns. We also found that SGN activity patterns are dysregulated in the absence of P2X3. These data show that calcium transients in embryonic SGNs are mediated by P2X3 receptors. Previously, extracellular ATP and purinergic receptors have been implicated in spontaneous calcium action potentials to organize neural circuits. Taken together, we conclude that SGN maturation requires P2X3-mediated extracellular ATP and intracellular calcium signaling.

P2244/B494

Cyclic AMP-dependent Co-stabilization of Axonal Arbors from Adjacent Developing Neurons.

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The connectivity of many neuronal networks is exuberant early during development, before axonal arbors are refined by activity-dependent competitive mechanisms. Competition is generally considered to entail synapse destabilization and pruning mechanisms resulting from asynchronous activity between pre- and post-synaptic partners. In addition, theoretical predictions include non-competitive interactions between co-active neighboring axons to co-stabilize their connections. The demonstration of such interactions in a physiological context has been limited by the complexity of the experimental paradigms

needed. We provide direct evidence that axonal arbors from neighboring retinal ganglion cells in the retina co-stabilize their branches. This co-stabilization influences axon pruning in the thalamus *via* a cAMP-dependent mechanism that does not affect spontaneous activity in the retina.

P2245/B495

Mechanistic Insights into the Interactions of Ndel1 with Neuronal Specific an kyrins and Implications in Axonal Polarity Maintenance through Selective Cargo Transport.

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Ankyrin-G (AnkG), a highly enriched scaffold protein in the axon initial segment (AIS) of neurons, plays crucial roles for the maintenance of axonal polarity and integrity of the AIS organization. In the AIS, an kG regulates selective intracellular cargo trafficking between the soma and axon through interaction with a dynein regulator Ndel1. However, the molecular mechanism governing the interaction of an kG with Ndel1 remains elusive. Here, we report that Ndel1 C-terminal coiled-coil (CT-CC) binds to both an kG and an kB (the homology of an kG) on the neuronal specific domains with a 2:1 molar stoichiometry. Furthermore, we solved high-resolution crystal structure of an kB in complex with Ndel1 CT-CC, revealing the detailed molecular basis for the complex assembly. Mechanistically, an kB binds with Ndel1 through forming a stable four-helix bundle majorly mediated by extensive hydrophobic interactions at six different layers. Moreover, we demonstrated that AIS cluster of Ndel1 required the specific interaction between an kG and Ndel1. Finally, we evaluated the effect of cargo sorting through the AIS by disrupting the an kG/Ndel1 complex. Collectively, our findings provide mechanistic insights into complex formation of an kG with Ndel1 and maintenance of axonal polarity and are valuable for further understanding of the functional roles of an kB/Ndel1 complex in cargo transport at the distal axon.

P2246/B496

Development of Corticospinal Tract Connections: a Light Sheet an alysis.

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The correct development of the nervous system depends on the establishment of precise functional connections between neurons and their targets. The corticospinal tract (CST) is a major descending motor pathway which originates in the cerebral cortex, projects through the internal capsule, cerebral peduncle and medulla, crosses at the level of the pyramids and terminates at different levels of the spinal cord. The molecular mechanisms underlying the functional development of the CST remain ill understood, partly due to difficulties in its retrograde tracing and 2D microscopic imaging. Our objective is to understand the mechanisms of CST development in mice at increased temporal and spatial resolution. To this end, we here combined novel techniques for tissue clearing, light sheet imaging and 3D reconstruction. We first optimized tissue clearing techniques for mouse brain and spinal cord of embryonic, early postnatal and mature adult stages. We then used UCHLl1:GFP reporter mice to visualize the CST and fluorescent Hb9 and Chat reporter mice to visualize brainstem and spinal motor neuron cell bodies and axons. Light sheet imaging was done with a Zeiss Z.1 microscope using 2.5 or 5x imaging objectives (EC Plan-Neofluar NA 0.16), a 5x LightSheet objective (LSFM 5x NA 0.1), a Clarity-

specific chamber (RI 1.45) and single-view acquisition mode. Image analysis and nerve desegmentation were performed using Imaris software. Our data show that CST, brainstem and spinal neurons can be monitored over time at single cell resolution allowing refined analysis of their growth, pathfinding, branching and connection. We conclude that this approach offers new perspectives to better understand the development of the motor nervous system and potentially also to apprehend its degeneration in ALS, primary lateral sclerosis and other diseases.

P2247/B497

Identification of Novel Downstream Targets and Function in Neurite Formation for Plxdc1.

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Neural circuits undergo neurite outgrowth in response to seizures, leading to aberrant axonal sprouting and changes to the morphology and connectivity of mature neurons. Thus, neurite growth inhibitory factors could have clinical applications. Identification of new therapeutic targets for epilepsy patients is crucial, as only about one third of patients can control seizures using current antiepileptic drugs. Previously, microRNA targeting Plxdc1 (Plexin domain containing 1) has been shown to be inversely correlated with epileptogenesis. Plxdc1 serves as a receptor for nidogen-1 and PEDF. Disruption of nidogen-1 leads to seizure-like symptoms, while deletion of PEDF may contribute to the etiology of Miller-Dieker Syndrome which is strongly associated with epilepsy. Interestingly, we observed that Plxdc1 overexpression (OE) drives the formation of neurite-like extensions in N2a cells. Plxdc1 OE in primary cortical neurons led to increased dendrite length, while Plxdc1 knockdown led to fewer and shorter dendrites. We confirmed these phenotypes in *vivo* by using in *utero* electroporation. To elucidate the Plxdc1 signaling pathway and identify potential therapeutic targets, we conducted a high-throughput kinase inhibitor screen in which N2a cells undergoing Plxdc1-OE-driven neurite formation were treated with 493 kinase inhibitors. The screening implicated Akt and its downstream target GSK3β in Plxdc1-OE-driven neurite formation, which we confirmed using primary cortical neurons. To identify a target of Plxdc1 that may directly interact with the cytoskeleton, we performed sub-cellular fractionation in N2a cells undergoing Plxdc1-OE-driven neurite formation. Proteomic analysis of the cytoskeletal fraction revealed a 3.43 fold increase in activity-dependent neuroprotector homeobox protein (Adnp) expression following Plxdc1 OE. Adnp interacts with microtubules and promotes microtubule assembly and polymerization. Plxdc1 signaling including Akt and GSK3β may influence neuronal morphology by regulating microtubules via Adnp; however, the precise mechanism should be investigated. We have identified a novel function for Plxdc1 in regulating neurite formation and propose several downstream targets for Plxdc1, which may have therapeutic roles in treating epilepsy. Further work is necessary to determine how changes in Plxdc1 expression impact neuronal connectivity and networks.

P2248/B498

Gq-mediated Calcium Dynamics and Membrane Tension Modulate Neurite Plasticity.

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The formation and disruption of synaptic connections during development is a fundamental step in neural circuit formation. Subneuronal structures such as neurites are known to be sensitive to the level of spontaneous neuronal activity but the specifics of how neurotransmitter-induced calcium activity

regulates neurite homeostasis are not yet fully understood. In response to stimulation by neurotransmitters such as acetylcholine, calcium responses in cells are mediated the Gαq/phospholipase Cβ (PLCβ)/ phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) signaling pathway. Here, we show that prolonged Gαq stimulation results in the retraction of neurites in PC12 cells and rupture of neuronal synapses by modulating membrane tension. To understand the underlying cause, we dissected the behavior of individual components of the Gαq/PLCβ /$PI(4,5)P_2$ pathway during retraction, and correlated these to the retraction of the membrane and cytoskeletal elements impacted by calcium signaling. We developed a mathematical model that combines biochemical signaling with membrane tension and cytoskeletal mechanics, to show how signaling events are coupled to retraction velocity, membrane tension and actin dynamics. The coupling between calcium and neurite retraction is shown to be operative in the C. *elegans* nervous system. This study uncovers a novel mechanochemical connection between the Gαq/PLCβ /$PI(4,5)P_2$ pathway that couples calcium responses with neural plasticity.

P2249/B499

Uridine Promotes Neurite Outgrowth in Neuro2a Cells.

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Neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease are the main causes of age-related dementia. These diseases can be due to neuronal cell death and impairment of neurite outgrowth. Giant oyster mushroom (GOM), *Pleurotus giganteus*, is used as a nootropic that can prevent the onset of dementia. The underline mechanism behind the medicinal property of GOM is unclear. Previous studies have shown that GOM has a high concentration of uridine. In this study, we examined the effects of uridine on neurite outgrowth in Neuro-2a (N2a) neuroblastoma cell line. In preliminary studies, we examined the effects of various concentrations of uridine on neurite outgrowth in N2a cells. When exposed to uridine N2a cells produced significantly longer neurite extensions ($P<0.001$) and exhibited a significant increase in neurite bearing cells ($P<0.001$) with an ideal concentration of 100M. Our preliminary results suggest that uridine significantly promoted neurite outgrowth in N2a cells. ($P<0.001$) Future studies are required to identify the mechanism behind uridine therapeutic potential on neurodegenerative diseases.

P2250/B500

Regulation of the Retromer Complex by Mtor Kinase.

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Introduction: mTOR kinase is an important regulator of neuronal cell development and neuronal plasticity. Among stages of neurogenesis regulated by mTOR is dendritogenesis. Primarily mTOR was believed to affect neurogenesis almost exclusively by controlling translation but now it is known that there are many other cellular processes downstream of mTOR involved in this process. Our preliminary data revealed that among mTOR targets is TBC1D5, a Rab7 GAP that regulates retromer activity. The retromer sets up the transport of proteins, including several membrane receptors, from the endosomes to the Golgi, known as retrograde transport. Retromer was shown to participate in dendritogenesis. Thus, we tested a hypothesis that mTOR regulates dendritogenesis at least partly via control of retromer

function.Materials and Methods. TBC1D5 phosphorylation was assessed by mass spectrometry and kinase assays in HEK293T cells. An alysis of TBC1D5 phosphorylation via mTOR was shown using stable HeLa cell lines - GFP versus TBC1D5-GFP WT or its mutants. Retromer function was tested with STxB uptake assay and immunofluorescence analysis of cellular VPS35 distribution in HeLa cells. Effect of mTOR-dependent TBC1D5 and VPS35 function on neuronal dendritic arborization and dendritic trafficking was tested in cultured hippocampal neurons.Results. We show that blocking mTOR activity with rapamycin influences retromer function. Upon treatment with rapamycin, fluorescently labeled STxB reached the Golgi at a decreased rate, suggesting an interplay between mTOR and the retromer. Our subsequent search for possible mTOR targets has found TBC1D5, a negative regulator of the retromer, to be phosphorylated by mTOR. The overexpression of unphosphorytable mutants of TBC1D5 or silenced VPS35 impaired attachment of retromer complex to the endosomal membrane as well as resulted in a significant simplification of dendritic arbors of developing neurons (observed effects are reversed by upstream mTOR regulator - PI3 kinase or an agonist of TrkB receptor - BDNF) and TrkB receptor transport along dendrite is changed.Discussion.The results of this study have revealed the mTOR kinase as a potential regulator of the retromer complex and contribution of the mTOR-retromer pathway in neuronal development. Moreover results point to intracelluar transport of TrkB receptor as key retromer-dependent processcritical for dendritogenesis.This work has been supported by Polish National Science Centre OPUS grant 2017/27/B/NZ3/01358.

P2251/B501

Retrograde Ret Signaling Controls Sensory Pioneer Axon Outgrowth.

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The trafficking mechanisms and transcriptional targets downstream of long-range neurotrophic factor ligand/receptor signaling that promote axon growth are incompletely understood. Zebrafish carrying a null mutation in a neurotrophic factor receptor, Ret, displayed defects in peripheral sensory axon growth cone morphology and dynamics. Ret receptor was highly enriched in sensory pioneer neurons and Ret51 isoform was required for pioneer axon outgrowth. Loss-of-function of a cargo adaptor, Jip3, partially phenocopied Ret axonal defects, led to an accumulation of activated Ret in pioneer growth cones, and reduced retrograde Ret51 transport. Jip3 and Ret51 were also retrogradely co-transported, ultimately suggesting Jip3 is a retrograde adapter of active Ret51. Finally, loss of Ret reduced transcription and growth cone localization of Myosin-X, an initiator of filopodial formation. These results show a specific role for Ret51 in pioneer axon growth, and suggest a critical role for long-range retrograde Ret signaling in regulating growth cone dynamics through downstream transcriptional changes.

P2252/B502

TRIM9 and TRIM67: Master Regulators of Developing and Adult-born Neurons.

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We have identified TRIMs 9 and 67 as two critical neuronally-enriched E3 ubiquitin ligases that regulate membrane remodeling & cytoskeletal dynamics during neuronal morphogenesis. They are essential for appropriate morphogenesis of murine embryonic cortical neurons at steady state & for appropriate

responses to the extracellular guidance cue netrin-1. TRIM9 is required for appropriate morphogenesis, migration, & synaptogenesis in adult-born neurons of the dentate gyrus. Deletion of either *Trims* results in subtle neuroanatomical defects, yet striking behavioral deficits, including spatial learning & memory deficits. These results indicate that TRIMs 9 & 67 are master regulators of form & function of developing embryonic & adult-born neurons. Our longterm goal is to understand how these ligases are uniquely positioned to temporally & spatially modulate neuronal morphogenesis via regulation of cytoskeletal dynamics & membrane trafficking. Based on previous studies from our lab we hypothesized that TRIMs 9 & 67 modulate neuronal morphogenesis via their ligase activity & by protein interactions. To identify additional interacting partners and/or ubiquitinated substrates, we performed a two-pronged unbiased proteomic study to identify additional putative interaction partners & ubiquitination targets of TRIM9 and TRIM67 from murine embryonic cortical neurons - the proximity-dependent biotin identification (BioID) approach & a ubiquitinated peptide enrichment approach. The two approaches identified numerous candidate interaction partners and/or substrates. Using immunoprecipitation & ubiquitination assays we validated some of the high priority candidates such as Myo16, Coronin-1A, Doublecortin, SNAP47, PRG-1, Tmod3, & Kinesin 3 family members KIF1a & b. Additionally, for a subset of these validated candidates, we used time-lapse TIRF microscopy & showed that they colocalize with the TRIM proteins.

P2253/B503

Early Development and Polarity Establishment in a Sensory Neuron Follow Pre-established Epithelial Apicobasal Polarity.

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Neurons are highly polarized cells with distinctive dendritic and axonal processes as well as their underlying microtubule polarity. A fundamental question is how axon-dendrite polarities are established in developing neurons during the development of nervous system. Here, we describe stereotyped development of a ciliated mechanosensory neuron, PDE, in *C. elegans*. PDE neuron is generated postembryonically from an epithelial lineage. Consistent with its epithelial origin, PDE neuron is born with apicobasal polarity, forming apical junctions with surrounding epithelial and sister neural cells. Using 4-D microscopy, we show that PDE neuron goes through apical constriction and its apical attachment remains anchored to the epithelia, marking the future site of ciliogenesis. Moreover, we find that an apical microtubule-organizing center (MTOC) is established following the apical constriction. Consistent with the nascent apical MTOC, γ-TuRC components translocate from centrosome to the site of apical attachment in PDE neuron. These results suggest that minus-end out microtubule polarity in the dendrite is likely laid out by the apical MTOC, which in turn may be established by PDE apical constriction. Based on these observations, we hypothesize that axon-dendrite polarity in PDE neuron may be derived from pre-existing apicobasal polarity from its epithelial lineage. Future investigation on the function of apicobasal polarity regulators during PDE development should reveal whether apicobasal polarity indeed instructs axon-dendrite polarity.

Establishing and Maintaining an Organelle 3

P2254/B505

Local Regulation of Lipid Synthesis Controls ER Sheet Insertion into Nuclear Envelope Holes to Complete Nuclear Closure.

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The metazoan nuclear envelope (NE) forms from bilayer glycerophospholipids synthesized in the ER that wrap around segregated chromosomes. To complete NE formation, ESCRT-III machinery transiently accumulates at NE holes that intersect with spindle microtubules and coordinates spindle disassembly with membrane fission. Here, we demonstrate that local control of glycerophospholipid synthesis serves as an additional mechanism to ESCRT-mediated NE closure by regulating ER sheet biogenesis. After meiosis II in C. elegans oocytes, ESCRTs accumulate into a plaque at the nascent NE adjacent to the dissipating meiotic spindle. However, nuclei are not detectably leaky in the absence of the NE-specific adaptors for ESCRT-III, LEM2 and CHMP7, suggesting that additional mechanisms contribute to NE closure. 3D electron tomography of meiotic oocytes revealed ER sheets that contact the outer nuclear membrane and feed into ~100 nm holes occluded by persisting spindle microtubules. Because the abundance of ER sheets proximal to the NE is controlled by CNEP-1 - the NE-enriched phosphatase that activates the key lipid metabolizing enzyme, lipin, to locally regulate glycerophospholipid synthesis - we tested the ability of cnep-1 mutant embryos to form sealed NEs. cnep-1 mutant embryos contain excess ER sheets that extend into the nuclear interior during NE formation and are leaky after both meiosis and mitosis. Nuclear morphology and sealing defects are also present in embryos depleted of lipin and are exacerbated upon deletion of LEM2 or CHMP7 indicating that local conversion of glycerophospholipids by lipin provides a partially overlapping mechanism with ESCRT-III activity to seal the NE. Preventing the formation of ectopic ER sheets by restoring glycerophospholipid synthesis in cnep-1 mutants rescues nuclear sealing defects. We conclude that ER sheets, limited by local control of glycerophospholipid synthesis, feed into NE gaps that intersect with spindle microtubules, and thus serve as a partially redundant mechanism to ESCRT-mediated membrane fission to ensure the closure of NE holes after open mitosis and meiosis.

P2255/B506

A Futile Cycle Initiated by ER-PM Tethering Drives Energy Expenditure.

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Spatial and temporal organization of signaling platforms generate biological signals encoded in amplitude or frequency. One such scenario involves calcium oscillations initiated from the ER-PM contacts. Here we show that the evolutionarily conserved Ral pathway balances energy metabolism and couples oscillatory calcium signaling via modulating ER-PM tethering. The RalGAP complex controls the activity of the G protein RalA, which in turn mobilizes the exocyst complex, a molecular tether. Liver-specific deletion of RalGAP, resulting in RalA activation, reduces circulating nutrients including lipids and glucose. Genetic and imaging analysis reveals that RalGAP inhibition accelerates the rhythmic frequency of calcium oscillations via increased PM-ER tethering. The increased phasic calcium signal promotes mitochondrial oxidative metabolism and fuel consumption, constructing a “futile cycle” of energy

expenditure. Hepatic RalGAP inactivation accordingly leads to an overall improvement in glucose and lipid metabolism during energy overload, alleviating pathologies including liver steatosis, obesity and insulin resistance. Collectively, our work reveals metabolic function resulted from modulating the ER-PM contacts, uncovering RalGAP as a molecular brake in energy metabolism via fine-tuning digitized cell signaling.

P2256/B507

Highspeed GI-TIRF-SIM Microscopy Reveals Extensive Co-assembly of Vimentin Intermediate Filaments with Peripheral ER-matrices.

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Vimentin intermediate filaments (VIFs) form an extensive intracellular network that coordinates a diverse list of cellular functions including the regulation of cell shape, force transmission, cytoplasmic mechanics and organelle positioning. Previous work has shown that VIFs surround and tether multiple organelles, including mitochondria, Golgi, lipid droplets, pigment granules and the nuclear envelope. However, if and how vimentin associates with the endoplasmic reticulum (ER), one of the largest and most morphologically complex organelles in the cell, is unknown. Here, using STED, 3D SIM, and grazing incidence TIRF-SIM (GI-TIRF-SIM) microscopy, we find that VIFs form extensive contacts with the ER. In COS-7, U2-OS, and mouse embryonic fibroblasts (MEFs), we observe VIF densities, or “knots,” that co-assemble with and stabilize highly concentrated matrices of ER tubules. Using an adaptive resolution multi-orientation segmentation algorithm, we find that VIF knots remain stably associated with ER matrices as both structures undergo persistent retrograde flow toward the perinuclear region. In peripheral regions devoid of assembled VIFs, we note a clear absence of ER matrices. Thus, we find that VIF knots represent a stable molecular marker for dynamic ER matrices, structures that were previously characterized only by their morphological complexity. Though vimentin frequently co-localizes with other cytoskeletal filaments, we do not observe enhanced F-actin or microtubule density at ER matrices. These results suggest one of three models: that VIFs pattern the morphology and distribution of ER, that ER matrices template VIFs, or both.

P2257/B508

Dual Role of Orp5 in Regulating Lipid Transport and Calcium Import to Mitochondria at Endoplasmic Reticulum (er)- Mitochondria Membrane Contact Sites.

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Mitochondria are dynamic organelles essential for cell survival whose structural and functional integrity rely on selective and regulated transport of lipids from/to the endoplasmic reticulum (ER) and across the

two mitochondrial membranes. As they are not connected by vesicle transport, the exchange of lipids between ER and mitochondria occurs at sites of close organelle apposition called membrane contact sites. However, the mechanisms and proteins involved in these processes are only beginning to emerge. We have found that ORP5/8 mediate non-vesicular transport of Phosphatidylserine (PS) from the ER to mitochondria in mammalian cells. We have also found that ER-mitochondria contacts where ORP5/8 reside are physically and functionally linked to the Mitochondrial Intermembrane space Bridging (MIB) complex, that anchors the outer and inner mitochondrial membranes, to facilitate PS transfer from the ER to the mitochondria. Finally, we show that ORP5 but not ORP8, additionally regulates import of calcium to mitochondria and consequently cell senescence.

P2258/B509

Loss of Mitochondria-Plasma Membrane Tethering Adversely Impacts Organelle Function and Cellular Fitness.

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The specific shape and positioning of mitochondria are intimately tied to the ability of this organelle to engage in its essential functions within the cell. The proper distribution of mitochondria contributes to the formation of a dynamic, interconnected network that is required for overall cellular fitness. Central to maintaining this network is the ability of mitochondria to associate with other organelles through membrane contact sites (MCSs), where tether proteins bring membranes into close proximity without fusion. Despite the importance of MCSs for the maintenance of cellular function, little is known about the mechanisms by which these MCSs contribute to organelle function and cellular fitness. Using *Saccharomyces cerevisiae* as a model system to investigate MCS function, we focus on Num1, the core component of the Mitochondria-ER-Cortex an chor, which has dual anchoring functions: tethering mitochondria at the plasma membrane (PM) for proper distribution of the organelle and anchoring the motor protein, dynein, to the PM, which is critical for nuclear inheritance during cell division. In addition to Num1's role as a physical anchor, our research suggests that Num1 plays a more direct role in maintaining mitochondrial function. Specifically, in the absence of Num1, cells have a growth defect when forced to respire, and preliminary data suggest Δ*num1* cells exhibit a decreased rate of oxygen consumption. The growth defect is distinct from other mutants which impact mitochondrial morphology and from Num1's role in dynein-mediated nuclear inheritance. Interestingly, this defect can be rescued by artificially clustering the mitochondrial interacting domain of Num1 at the PM, but not by tethering mitochondria to the PM with synthetic tethers that lack domains of Num1. These data suggest that there are Num1-dependent functions of mitochondria-PM contacts, beyond physically tethering the two membranes, that impact mitochondrial and cellular functions. These studies highlight the idea that MCSs are not simply sites of physical tethering between membranes, but functional hubs that are critical for overall cellular fitness.

P2259/B510

A Mitochondrial Translocon Subunit Promotes Assembly of the Cristae Organizing MICOS Complex in Proximity to ER Contact Sites.

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Mitochondrial cristae organization is regulated by the highly conserved MICOS complex. MICOS, an approximately megadalton complex comprised of six conserved subunits, localizes to cristae junctions, sites of membrane invagination on the mitochondrial inner membrane. The principal MICOS component, Mic60, assembles into structures that persist in the absence of other complex members and in mitochondria devoid of cristae, suggesting it marks sites of MICOS assembly and cristae biogenesis. However, there are no known upstream determinants of Mic60 assembly and positioning. Using an unbiased visual screen, we find that a subunit of the translocase of the outer mitochondrial membrane (TOM) complex regulates Mic60 assembly into structures that predominantly localize in proximity to endoplasmic reticulum (ER) membrane contact sites. Mic60 assemblies are more generally linked to inter-organelle contact sites as they reposition in proximity to mitochondrial-vacuole contacts in a yeast strain where the ER-mitochondrial tether is deleted and bypassed. These data reveal that positioning of the MICOS complex is contingent on extra-mitochondrial spatial cues and are consistent with a model that a phospholipid micro-environment on the inner mitochondrial membrane generated by inter-organelle contact sites regulates MICOS assembly and dictates cristae biogenesis sites.

P2260/B511

ER Membrane Contact Sites Are a Platform for Regulating Mitochondrial Dynamics and Bioenergetics.

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Mitochondria are organelles derived from ancient endosymbiotic organisms and are present in the cytoplasm of all eukaryotes. Despite the fact that they are rapidly fusing and dividing, they are somehow able to maintain a characteristic morphology. Disruption of this steady-state morphology results in either a fragmented or elongated network, both of which are associated with altered metabolic states and disease. How cells spatially organize division and fusion machineries to produce mitochondria of appropriate size is a fundamental question that is still unresolved. We now know that different organelles form membrane contact sites with each other to coordinate their function in the cellular context and mitochondria form conspicuous contact sites with the endoplasmic reticulum (ER MCS). These ER MCS regulate calcium buffering, lipid transfer, and mark the position of division along the length of the mitochondrion. Here we show that ER MCS also mark the position of mitochondrial fusion using a photoconvertible fluorescent protein targeted to mitochondrial compartments allowing the detection of content exchange upon a *bona fide* fusion event. Surprisingly, we found that both division and fusion machinery (Drp1 and Mitofusins) accumulate together in nodes at ER MCS where mitochondria can perform either process. Additionally, depolarization of individual mitochondria revealed that fusion at ER MCS nodes rescues the polarization state of the large majority of depolarized mitochondria. Thus, we propose that division and fusion are spatially coordinated at ER MCS where mitochondria are poised to alter their morphology in response to metabolic needs.

P2261/B512

Single Molecule Characterization of Protein Dynamics at Endoplasmic Reticulum-Organelle Contact Sites.

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The endoplasmic reticulum (ER), present in all eukaryotic cells, consists of a complex membranous network stretching from the nuclear envelope to the plasma membrane. This remarkably versatile organelle plays crucial roles in numerous cellular functions, from calcium signaling to protein and lipid synthesis. Recent work has also highlighted an emerging role for the ER as a key regulator of the morphology and function of other subcellular organelles, with ER-organelle contact sites mediating the exchange of crucial signaling components and biomolecules. Several candidate proteins have been proposed to act as molecular tethers to facilitate specific ER-organelle interactions. However, the dynamics and spatial regulation of these tethering proteins within the ER membrane remains unclear. Here, we introduce a high-speed, correlative single molecule imaging approach to study molecular behavior at ER-organelle contact sites. With this approach, we track the motion of several ER-organelle tethering proteins in live cells, including VAP-A, VAP-B, Mfn1, Mfn2, and PDZD8. We demonstrate that each tether exhibits a unique dynamic signature and spatial confinement pattern, with tethering complexes assembling and disassembling on subsecond timescales. Based on these measurements, we conclude that distinct subtypes of contact site exist, and can be grouped according to the dynamic properties of specific tethers. We observe heterogeneity in tethering protein dynamics across different cell types and at spatially discrete ER-organelle contacts within the same cell. Furthermore, several tethering proteins exhibit specific alterations to their dynamic behavior in response to nutrient deprivation or ER calcium depletion, suggesting that the physiological state of cells impacts ER-organelle contacts by changing tethering complex dynamics. Fascinatingly, we find that molecular tethers harboring certain disease-associated mutations demonstrate altered dynamics at the single molecule level, with this impaired behavior correlating with downstream cellular pathogenesis. Taken together, this work provides initial insights into the single-molecule dynamics of tethering proteins at ER-organelle contact sites. Alterations in these dynamics appear to be a key component in dictating the functional heterogeneity of contact sites, and potentially underlie certain ER-associated disease states.

P2262/B513

Regulation of Endoplasmic Reticulum Architecture by the Er-shaping Protein Climp63.

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The Endoplasmic Reticulum morphology is structural defined by ER-shaping proteins and divided in functional compartments, which include, the nuclear envelope, rough ER (predominantly sheet-like cisternae decorated by membrane-bound ribosomes) and smooth ER (interconnected tubular network that spans to the cell periphery). ER sheets constitute flat surfaces that accommodate ER-ribosome interactions which contribute to the biosynthesis of secretory proteins, and ER-microtubule links that regulate ER morphology. Regulation of ER architecture and interactions with Ribosomes or Microtubules can be modulated by sheet-promoting proteins such as CLIMP63. However, the molecular mechanisms that coordinate sheet formation, dynamics and functional specificity are not understood. Here, we have

characterised the effect of CLIMP63 post-translation modifications in ER morphology and interactions with different cellular components. We have determined enzymes that regulate CLIMP63 stability and uncover novel CLIMP63 regulators which affect both ER-architecture and function.

P2263/B514

Isoform-specific Differences in the Function of Hereditary Spastic Paraplegia-associated Protein Spastin in Endoplasmic Reticulum Morphogenesis and Cellular Homeostasis.

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Hereditary spastic paraplegias (HSPs) are a genetically diverse group of inherited neurodegenerative disorders selectively affecting the longest corticospinal motor neurons; they are characterized most prominently by the progressive spasticity of the lower limbs. Molecular etiologies of HSPs are complex and caused by mutations in one of more than 60 identified genes. However, the most common forms, representing about half of all affected patients, are due to autosomal dominant mutations in one of three tubular ER-shaping proteins: spastin, atlastin-1, and REEP1. Therefore, ER-shaping defects appear to be a common pathogenic mechanism, however, it is not known how ER morphology defects contribute to degeneration and dysfunction of corticospinal motor neurons. Mutations in the *SPAST* gene are the most common causes of HSP, and the *SPAST* gene product spastin is a microtubule-binding and -severing AAA ATPase protein. A single *SPAST* mRNA transcript produces two isoforms (M1 and M87) with different subcellular localizations and functions. The full-length M1 isoform contains an N-terminal 86-amino acid domain that is absent in the shorter M87 isoform. This N-terminal region harbors a membrane-bound hydrophobic hairpin that confers ER localization and generates membrane curvature to shape the tubular ER. The more abundant M87 spastin is largely cytosolic and has been functionally implicated in endosomal trafficking and cytokinesis. While the overwhelming majority of mutations in *SPAST* will affect the expression of both isoforms, it is not known which isoform contributes to HSP pathogenesis. Here, we have used CRISPR/Cas9 gene editing to generate isoform-specific knockout cell lines. Knockout of M1 spastin results in aberrant peripheral ER morphogenesis and loss of tubular ER polygonal networks, indicating that M1 is necessary for proper tubular ER formation and organization. Isoform-specific roles of spastin in ER morphogenesis and dynamics were also examined in cells specifically expressing each spastin isoform, as well as spastin isoforms harboring the ATPase defective, HSP-associated mutation K388R. Expression of M1 spastin K388R but not M87 spastin K388R results in ER membranes trapped on microtubules. Furthermore, fluorescence recovery after photobleaching (FRAP) and single-molecule imaging approaches were used to examine the specific dynamic nature of each spastin isoform. Understanding the functional contributions of each isoform will be crucial for understanding the etiology of HSP and for finding new therapeutic targets.

P2264/B515

Microtubule Polyglutamylation Controls Organelle Distribution.

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Organelle distribution plays central roles in cell function. While microtubule plays key roles in organelle distribution, how microtubule modification affects this function is largely unknown. Here, we show that three endoplasmic reticulum (ER) membrane proteins, Climp63, p180 and Kinectin preferentially binds centriolar, perinuclear and peripheral microtubules, respectively, to maintain a balanced distribution of

the perinuclear ER. We further show that p180 and Kinectin do so by specifically binding mono- and poly-glutamylated microtubules, respectively. Depletion of Climp63, KTN or p180, or manipulating microtubule populations or glutamylation status dramatically changed the distribution of the ER, especially perinuclear ER. We further show that this change in ER distribution also leads to broad changes in the distribution of other organelles. Together, our data show that microtubule modification controls organellar distribution by regulating perinuclear ER morphology.

P2265/B516

Novel Transverse ER within Corticospinal Axons Revealed by FIB-SEM Reconstruction in a Hereditary Spastic Paraplegia Mouse Model.

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The ER is an interconnected network of narrow tubules broadly distributed within cells and their process, including along the entire axonal length. Hereditary spastic paraplegias (HSPs) are often caused by mutations in structural proteins of the ER, including atlastin-1 and Reep1. Here we report on the structural disruption of the ER by the introduction of double mutations in mice, knocking in atlastin-1-K80A (GTPase-defective) and knocking out Reep1. These double mutant mice show an HSP phenotype, with prominent hindlimb impairment. To find the mechanism underlying this phenotype, we examined the corticospinal tracts in spinal cord. With transmission electron microscopy (TEM), we found a dramatic expansion of ER in axons. To obtain a more complete view of ER changes in axons, 3D reconstructions were carried out. The corticospinal tract area (40x30x70 µm) at L5 was imaged by FIB-SEM at 6 nm isotropic resolution. Amira software was used for the 3D reconstruction. Unlike the wild-type mouse whose axonal ER is tubular with some branching, axonal ER of the mutant mouse is defined by regularly-interspersed, transverse matrix-like structures connected to each other via tubules. The tubular matrices occupy most of the cross-sectional area of the axon. The reason for the change in ER structure remains under investigation, but these tubular matrices are reminiscent those seen in other cell types (Nixon-Abell J, et al. Science 2016) and the tubules connecting these structures were of normal axonal ER diameter (20-30 nm) as reported previously (Terasaki M, J Cell Sci 2018).

P2266/B517

ReepA Is Required for Endoplasmic Reticulum Clearance from Chromosomes but Not Endoplasmic Reticulum Partitioning to Spindle Poles in Dividing *Drosophila* Cells.

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The endoplasmic Reticulum (ER) is essential for biogenesis of lipids, proteins, and steroid hormones, as well as calcium signaling. The ER cannot be formed *de novo* and must be inherited during cell division. In animal cells, ER inheritance depends on partitioning of the organelle to the poles of the mitotic spindle via direct association with astral microtubules, and defining the specific mechanisms that link the ER to spindle microtubules is therefore essential to our understanding of developmental and physiological processes that depend on ER functions. It was recently shown in mammalian cells that Receptor Expression Enhancing Proteins (REEPs) 3 and 4 are required for clearance of the ER from chromosomes in mitotic cells, via a mechanism that involves direct association of the ER with spindle microtubules and focusing of the ER around spindle poles. Thus, in addition to ensuring exclusion of the ER from nuclei,

REEP proteins may also play a role in microtubule and spindle pole-dependent ER inheritance in dividing cells. To test this in an animal model, we generated *Drosophila* with a targeted deletion of the *REEPA* gene, the fly orthologue of human REEPs 3 and 4. *REEPA* homozygous mutant flies were fully viable and fertile, suggesting *REEPA* is not essential for normal animal development or overall physiology. In *vivo* analysis of syncytial embryonic nuclear divisions and dividing spermatocytes revealed that ER partitioning to spindle poles and association with astral MTs were largely unaffected in *REEPA* mutants, suggesting ReepA is not required for ER inheritance in these tissues. Surprisingly, however, close examination revealed abnormal strands of ER within the spindle, extending from the spindle poles towards the aligned chromosomes, in both *REEPA* mutant embryos and spermatocytes. We also observed abnormal accumulations of ER that were directly associated with chromosomes in interphase nuclei of *REEPA* mutant embryos and spermatocytes. These findings are consistent with results from human cells with both REEPs 3 and 4 suppressed, suggesting a highly conserved mechanism whereby REEP proteins are required to sequester ER membranes away from chromosomes during cell division and prevent ER entrapment in the newly formed interphase nuclei. Importantly though, our findings also suggest that REEP proteins are not the key factors that universally associate the ER with spindle microtubules and ensure proper partitioning of the organelle to daughter cells.

P2267/B518

Endomembrane Surfaces Regulate a PolyQ-Dependent Phase Transition.

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Compartmentalization of molecules into subcellular organelles is essential for life. Biomolecular condensates, composed of phase-separated protein and nucleic acid, are important centers of compartmentalization in diverse contexts. Phase separation is driven by weak, multivalent interactions, resulting in the formation of condensed structures that can display properties of liquid-like droplets. Although the ability to phase separate is an intrinsic feature of many proteins and nucleic acids, cells must regulate condensation to assemble functional structures at the appropriate time and location. The regulatory mechanisms that govern when and where condensates form are unknown. Our group discovered that biomolecular phase transitions play essential physiological roles in a multinucleate fungus (Zhang *et al., Mol Cell* 2015; Langdon *et al., Science* 2018). Specifically, the RNA-binding protein Whi3 undergoes a polyglutamine (polyQ)-dependent phase transition to generate distinct, functional condensates. These condensates form with different RNA transcripts that regulate either the cell cycle or cell polarity, and are positioned near nuclei or sites of polarized growth, respectively. How do cells control assembly and patterning of these different droplets in time and space? We hypothesized that Whi3 condensates may be patterned by endomembranes associated with nuclei and sites of polarity. Indeed, here we find that Whi3 condensates stably associate with the endoplasmic reticulum. Moreover, we find that membrane surfaces dramatically reduce the concentrations of Whi3 and RNA required to drive phase separation in *vitro* compared to free-diffusing molecules in solution. These results suggest that endomembranes regulate Whi3 condensation by restricting molecular diffusion to a two-dimensional surface. How are condensates recruited to endomembranes in cells? We find that a molecular chaperone complex associated with the endoplasmic reticulum plays an important role in recruiting Whi3 to membranes. Further, this chaperone complex modifies the biophysical properties of Whi3 condensates, suggesting that the identities of membrane-associated condensates are determined by active cellular processes. Collectively, these findings provide key insight into the biophysical

mechanisms that govern the assembly, patterning, and emergent properties of biomolecular condensates.

Mitochondrial Physiology

P2268/B519

Proteomic Screening Identifies Novel Interactors of MICU1 Independent of the Mitochondrial Calcium Uniporter.

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MICU1 is an EF-hand-containing mitochondrial protein that gates the mitochondrial Ca^{2+} uniporter channel (mtCU) and directly interacts with the pore-forming subunit MCU. Previous reports suggest that genetic loss-of-function phenotypes associated with MICU1 deletion are not entirely mtCU dependent. This suggests that MICU1 may have cellular functions independent of its role in regulating the mitochondrial calcium uptake through the uniporter. To discern novel MICU1 functions, we employed a biotinylation-based proteomic approach in *Mcu*$^{-/-}$ and *Micu1*$^{-/-}$ cells to detect MICU1 interactors employing a fusion construct containing BioID2, a small biotin ligase for proximity-dependent labeling. Expression of MICU1-BioID2 in *Mcu*$^{-/-}$ cells, coupled with appropriate controls, allowed the identification of mtCU-independent interactors. The LC-MS analysis of biotinylated proteins after avidin-based purification identified the Mitochondrial Contact Site and Cristae Organizing System (MICOS) components IMMT, CHCHD2, and CHCHD3 as MICU1 interacting partners (MICU1-BioD2 expressed in Micu1$^{-/-}$ cells to avoid aberrant expression). These same MICOS components were identified in MCU$^{-/-}$ cells, suggesting that MICU1 may be involved in the regulation of MICOS independent of the mtCU. Fast protein liquid chromatography (FPLC), blue native-PAGE, co-immunoprecipitation, live-cell Ca^{2+} imaging, confocal and super-resolution imaging methods were used to confirm novel MICU1 interactions and sub-mitochondrial localization. Further, the deletion of the *Chchd2* resulted in the loss of MICOS and acute changes in cristae organization without any observable effect on $_{m}Ca^{2+}$ uptake. These results suggest that MICU1 likely serves cellular functions independent of the mtCU and may serve as a key sensor/regulator of Ca^{2+}-dependent signaling in other mitochondrial processes that are essential for cellular function.

P2269/B520

Insulin Receptor Preserves Mitochondrial Function by Binding VDAC1 in Insulin Insensitive Mucosal Epithelial Cells.

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Purpose: Maintenance of the corneal epithelium is essential for the preservation of vision. Unlike other epithelial cells, corneal epithelial cells (CECs) do not require insulin for glucose uptake, but do require insulin for mitochondrial respiration. In this study, we examined the role of insulin and insulin receptor (INSR) in mediating metabolic activity, mitochondrial stability and mitophagy in CECs and bronchial epithelial cells, a second mucosal epithelial cell type. Methods: Telomerized human corneal epithelial (hTCEpi) cells and bronchial epithelial cells (HBECs) were cultured in serum-free keratinocyte growth medium with (KGM) or without (KBM) supplements. Cells were treated with or without insulin for 24 or

48 hours. To examine the role of INSR, cells were transfected with siRNA oligonucleotides. Immunoprecipitation, mitochondrial fractionation, western blotting and immunofluorescence were used to determine the expression and subcellular localization of INSR and mitochondrial proteins. Mitochondrial morphology and function were analyzed by using mitotracker-TMRE staining and electron microscopy. Mitochondrial activity was measured using a Seahorse Metabolic Flux an alyzer. Results: in hTCEpi cells, 48 hours in KBM shifted cells towards a glycolytic phenotype that was associated with a loss of mitochondrial membrane polarization and a reduction in expression of mitochondrial proteins. In HBECs, KBM shifted cells to a more respiratory phenotype with no changes in mitochondrial polarization or protein expression. Treatment with insulin blocked these effects in hTCEpi cells, but further increased respiration in HBECs. INSR knockdown in hTCEpi cells also showed a reduction in mitochondrial protein expression and function, along with a decrease in mitophagy markers PINK1 and LC3-II. Surprisingly, INSR bound VDAC1 in mitochondria in hTCEpi cells. INSR was undetectable in mitochondria from HBECs. Conclusions: These data confirm differential roles for insulin and INSR in regulating metabolic activity in the corneal and bronchial epithelium. These findings further suggest that the INSR-VDAC1 interaction in the mitochondrial membrane is essential to maintain mitochondrial homeostasis in the corneal epithelium. Additional studies are needed to define the underlying mechanism(s) that mediate mitochondrial homeostasis in mucosal epithelia.

P2270/B521

Time-dynamics of Mitochondrial Membrane Potential Reveal an Inhibition of ATP Synthesis in Mitosis.

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The energetic demands of a cell are believed to increase during mitosis. Cellular ATP levels decrease from G2/M transition through metaphase-anaphase transition, consistent with elevated consumption. The rates of ATP synthesis during mitosis, however, have not been quantified. Here, we monitor mitochondrial membrane potential of single lymphocytes and demonstrate that cyclin-dependent kinase 1 (CDK1) activity causes mitochondrial hyperpolarization from G2/M until the metaphase-anaphase transition. By using an electrical circuit model of mitochondria, we quantify the time-dynamics mitochondrial membrane potential under normal and perturbed conditions to extract mitochondrial ATP synthesis rates in mitosis. We found that mitochondrial ATP synthesis decreases by approximately 50 % during early mitosis, when CDK1 is active, and increases back to G2 levels during cytokinesis. Consistently, acute inhibition of mitochondrial ATP synthesis failed to delay cell division. Our results provide a quantitative understanding of mitochondrial bioenergetics in mitosis and challenge the traditional dogma that cell division is a highly energy demanding process.

P2271/B522

Quantitative Variation in Mitochondrial 3243A>G Mutation Produce Discrete Changes in Energy Metabolism.

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Mitochondrial DNA (mtDNA) 3243A>G tRNALeu$^{(UUR)}$ heteroplasmic mutation (m.3243A>G) exhibits clinically heterogeneous phenotypes. While the high mtDNA heteroplasmy exceeding a critical threshold causes mitochondrial encephalomyopathy, lactic acidosis with stroke-like episodes (MELAS) syndrome, the low mtDNA heteroplasmy causes maternally inherited diabetes with or without deafness (MIDD) syndrome. How quantitative differences in mtDNA heteroplasmy produces distinct pathological states has remained elusive. Here we show that despite striking similarities in the energy metabolic gene expression signature, the mitochondrial bioenergetics, biogenesis and fuel catabolic functions are distinct in cells harboring low or high levels of the m.3243A>G mutation compared to wild type cells. We further demonstrate that the low heteroplasmic mutant cells exhibit a coordinate induction of transcriptional regulators of the mitochondrial biogenesis, glucose and fatty acid metabolism pathways that lack in near homoplasmic mutant cells compared to wild type cells. Altogether, these results shed new biological insights on the potential mechanisms by which low mtDNA heteroplasmy may progressively cause diabetes mellitus.

P2272/B523

CoA Biosynthesis Enzyme Localization and PANK2 Loss of Function Studies Indicate Compartmentalized CoA Synthesis Is Required for Metabolic Switching to β-oxidation.

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Pantothenate kinase (PANK) performs the rate-limiting step in Coenzyme a (CoA) biosynthesis. There are four catalytically active PANK protein isoforms: PANK1α, PANK1ß, PANK2 and PANK3, each with different subcellular localizations. Mutations in the *PANK2* gene cause the disease Pantothenate Kinase Associated Neurodegeneration (PKAN). Organelle-specific acetyl-CoA concentrations are known to regulate metabolism in response to changing cellular conditions, recent data indicates that tightly regulated, compartmentalized CoA pools may exist as well. Here we show through cellular fractionation that not just PANKs, but a full complement of CoA biosynthetic enzymes exists in the cytosol, mitochondria, and nucleus. *De novo* organelle-specific CoA biosynthesis would allow for direct manipulation of local CoA and acetyl-CoA pools. In the mitochondria this could be necessary to meet the increased CoA demand during β-oxidation, while in the nucleus it would provide independent control over acetyl-CoA concentration regulating histone acetylation. PANK2 is known to be activated by acylcarnitines, abundant in high fat conditions, and it is the only PANK protein localized to both the

mitochondria and nucleus. Therefore, we hypothesize that PANK2 is required to meet the increased mitochondrial CoA demand during β-oxidation (compared to glucose respiration) and to support the *de novo* production of acetyl-CoA in the nucleus used for histone modification. To test these hypotheses, nuclear acetylation status and β-oxidation in PANK2-deficient cells vs. controls was studied in glucose vs. palmitate growth conditions. Control cells exposed to palmitate showed major changes in nuclear acetylation while PANK2 knockdown HEK293T cells did not respond to palmitate in the same manner. PKAN patient-derived fibroblasts (*PANK2* null) exhibited a diminished respiratory response when challenged with palmitic acid compared to control fibroblasts as measured by the Seahorse XF an alyzer. Our findings indicate that PANK2 is required to support metabolic shifts from glucose to fatty acid metabolism, suggesting that compartmentalized CoA synthesis is required for rapid shifts in cellular metabolism.

P2273/B524

Igfbp-3 Preserves Mitochondrial Function during Hyperosmolar Stress in Corneal Epithelial Cells.

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Purpose: Hyperosmolar stress induces mitochondrial dysfunction that results in corneal epithelial surface damage in patients with dry eye disease. The purpose of this study was to characterize the role of the insulin-like growth factor binding protein-3, IGFBP-3, in corneal epithelial cells exposed to hyperosmolar stress. Methods: Telomerase-immortalized human corneal epithelial (hTCEpi) cells were cultured in defined keratinocyte growth (with supplements) or basal (no supplements) media (330 mOsm). NaCl was added to bring osmolarity values up to 450 and 500 mOsm and cells were cultured for 6 or 24 hours with or without 500 ng/ml recombinant human (rh)IGFBP-3. Mitochondrial respiration, ATP production, and spare respiratory capacity was measured using a Seahorse Metabolic Flux an alyzer. Cell cycle was measured using a Celigo Imaging Cytometer. Intracellular IGFBP-3 was assessed using western blot and secreted IGFBP-3 was quantified using ELISA. Mitochondrial (mt)DNA was visualized using SYBR green staining and imaged using confocal microscopy. JC-1 staining was used to assess mitochondrial membrane polarization. Mitochondrial calcium levels were measured using Rhodamine (Rhod-2). Results: Hyperosmolar culture induced cell cycle arrest in G0/G1 at 6 hours. There was a decrease in mitochondrial calcium, membrane polarization, mitochondrial respiration and ATP production at 6 and 24 hours of hyperosmolar culture. Intra- and extracellular IGFBP-3 was attenuated at 24 hours. Supplementation with rhIGFBP-3 preserved mitochondrial respiration. Similarly, SYBR green staining was decreased at 450 mOsm. This decrease was not evident in cells treated with rhIGFBP-3. Conclusions: Taken together, these findings suggest that IGFBP-3 plays a critical role in cellular homeostasis during stress. Further studies are necessary to determine how IGFBP-3 mediates stress responses in the corneal epithelium.

P2274/B525

Role of ALS-linked UBQLN2 in Maintaining Mitochondrial Function.

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Amyotrophic Lateral Sclerosis (ALS) is a debilitating neurodegenerative disorder that induces fatality through the loss of motor neurons. ALS can be caused by mutations in ubiquilin-2 (UBQLN2), a protein

that maintains cellular proteostasis by binding ubiquitinated misfolded proteins and clearing them through the autophagy and proteasomal degradation systems. These functions help to deter protein aggregation, a hallmark pathological feature of ALS. However, the underlying mechanism by which UBQLN2 mutations cause ALS pathogenesis remain unclear. To discover the pathophysiologic mechanisms that cause disease, we generated transgenic mouse lines with neuron-specific expression of either human wild-type UBQLN2 (WT356 line) or a UBQLN2 ALS mutant (P497S line). The P497S mouse line, but not the WT356 line, developed motor neuron disease. Spinal cord and hippocampal tissues were isolated from these transgenic lines and used to generate a large-scale proteomics profile. Proteomics indicated a systematic downregulation of mitochondrial proteins in the lumbar spinal cords of the P497S mutant compared to the WT and non-transgenic lines. Transmission electron microscopy of spinal cord motor neurons showed significant structural deterioration of cristae in the mitochondria of the P497S mutant. As cristae are vital for oxidative phosphorylation and ATP production, mitochondrial function was likely impaired as well. Seahorse respiration assays on isolated mitochondria from the spinal cord and hippocampus indicated that mitochondrial state 3 respiration, a measure of ADP to ATP conversion, becomes severely perturbed as P497S mice age. To investigate the role of UBQLN2 in maintaining mitochondrial structure and function, we used CRISPR-Cas9 to generate HeLa and motor-neuron-like NSC-34 knock-out cell lines of UBQLN2. Inactivation of UBQLN2 caused an attenuation of mitochondrial respiration and ATP production, and this functional defect was further exacerbated by oxidative switching from glycolytic media (glucose) to oxidative media (galactose). These functional defects could be rescued through re-expression of WT UBQLN2 into the knock-out line. These studies suggest that UBQLN2 protein is required for proper maintenance of mitochondrial function and suggests that the ALS P497S mutant causes mitochondrial dysfunction through a loss-of-function mechanism.

P2275/B526

Structural Basis of Mitochondrial Coenzyme Q Biosynthesis by the Human Coq7-coq9 Complex.

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Coenzyme Q (CoQ) is a redox-active lipid that is essential for cellular respiration, and serves as a cofactor for numerous mitochondrial enzymes and extramitochondrial functions. Most eukaryotic CoQ is produced by the CoQ biosynthetic pathway, and a majority of the proteins that participate in CoQ production (COQ1-11) are located on the matrix face of the mitochondrial inner membrane. These proteins interact with each other in a highly cooperative manner, and form various subcomplexes and a high-molecular weight complex – complex Q – in mitochondria. However, the mechanistic details of this dynamic network of protein-protein interactions and complex Q assembly pathway is not well understood. Here, we report a 3.6 Å electron cryo-microscopy structure of the human COQ7-COQ9 biosynthetic complex. The structure reveals four copies of each molecule forming a hetero-octameric complex. We observe many charge-charge and hydrophobic interactions on three different molecular interfaces between the hydroxylase COQ7 and the lipid-binding COQ9. Our atomic models also show clearly discernible ligand densities inside the hydrophobic cavities of both molecules. These data provide novel insights into the molecular architecture of complex Q components, and a foundation for gaining mechanistic insights into the molecular functions that facilitate CoQ biosynthesis.

P2276/B527

Hypoxia-mediated Downregulation of Mitochondrial Pyruvate Carrier (mpc) through Interplay with Notch Signaling Pathway Prevents Cancer Cells from Hypoxia-induced Apoptosis.

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Mitochondrial pyruvate carrier (MPC), which is essential for mitochondrial pyruvate usage, mediates the transport of cytosolic pyruvate into mitochondria. Previous reports have shown that the MPC is a key regulator of glycolysis in tumor cells and reduced expression of MPC is associated with stemness of cancer cells. However, the mechanism by which MPC downregulated is largely unknown. In the present study, we found that MPC1 is transcriptionally downregulated under prolonged hypoxic conditions (1% O_2) in human hepatocellular carcinoma cell lines (Hep3B and Huh7). We also showed that Hey1 and Hey2, Notch signaling target genes, are necessary for the reduced expression of MPC. Furthermore, HIF-2α -dependent upregulation of DEC1(Stra13/Bhlhe40), the basic-helix-loop-helix transcription factor, is necessary for Hey1 and Hey2 expression but not sufficient for transcriptional repression of MPC1. We also elucidated that hypoxia-induced apoptotic cell death in PLC/PRL5 cell line, in which MPC expression did not change under hypoxia, was relieved upon MPC1 downregulation using siRNA or CRISPR/Cas9 system. We also showed that Hep3B cells which stably expressed MPC protein induced cell death only when they exposed to hypoxic condition. Taken together, the present data demonstrate that Notch signaling pathway under hypoxia suppresses pyruvate-dependent oxidative phosphorylation and promotes cancer cell survival through downregulating MPC expression. This work was supported by a National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MSIP) (NRF-2017R1A2B4007462, NRF-2017R1A2B4009674 and NRF-2011-0030086)

P2277/B528

The Ribonucleoprotein Clueless Affects the Metabolism by Regulating the Abundance of Enzymes Associated with Glycolysis and Respiratory Chain Components.

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The majority of cellular ATP is produced in mitochondria and functional mitochondria are vital for maintaining proper cellular homeostasis. In order to carry out their many biochemical functions, mitochondria must import approximately 1800 nucleus-encoded proteins. An y aberrations in these proteins or in the import mechanisms can cause a cascade of mitochondrial defects. Much is known about the canonical pathways involved in the import of nucleus-encoded proteins; proteins are translated by free ribosomes in the cytoplasm, bind chaperons and are targeted to the organelle. However, there is emerging evidence supporting co-translational import occurs, but the mechanism is not well-defined. We are investigating the role of the Drosophila ribonucleoprotein Clueless (Clu) in this process. *clu* mutants have defective mitochondria and the flies are sick and uncoordinated. Clu interacts with outer mitochondrial membrane proteins such as Tom20, Porin and PINK1. We have also shown that Clu binds mRNA and associates with ribosomal proteins. The mammalian counterpart, Cluh, was shown to preferentially bind nucleus-encoded mitochondrial mRNAs. We are currently determining Clu's molecular mechanism and whether Clu assists with mitochondrial protein import. We used immunoprecipitation followed by mass spec analysis to identify potential Clu interactors. We also analyzed the mitochondrial proteome from *clu, Sod2* and *Pink1* mutants using TMT mass spectrometry. These analyses reveal that Clu preferentially interacts, as expected, with the ribosomal proteins from both 60S and 40S ribosomal subunits as well as various core translation factors. Surprisingly, in addition

to proteins involved in translation, we found that Clu associates with glycolytic enzymes and several other metabolic enzymes. We confirmed that Clu binds to *Pyruvate kinase* and *Enolase* using reciprocal CoIPs. Moreover, we show that starvation causes Clu to dissociate from many of its potent interactors. Our TMT results show that a subset of OXPHOS complex members are specifically less abundant in *clu* mutants. We also confirmed that the changes in protein abundance are not due to altered transcription. Lastly, we found that the altered protein profile is *clu* specific, and not due to general mitochondrial damage, by comparing TMT from Sod2 and PINK1 mutants as controls. As a ribonucleoprotein Clu is expected to preferentially bind ribosomal proteins and core translation factors. But, surprisingly, Clu also associates with glycolytic enzymes and especially, the OXPHOS components, are severely reduced in *clu* mutants. Our data supports a model whereby Clu affects cellular metabolism by controlling the availability of enzymes associated with glycolysis and mitochondrial oxidative phosphorylation.

P2278/B529

Alcohol-induced Defects in Mitochondrial Structure and Dynamics Are Further Altered by Lipid Droplet Accumulation.

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Although the progression of alcoholic liver disease is well-described clinically, much less is understood about the molecular basis of the disease. Over a dozen years ago, we determined that microtubules are hyper acetylated in alcohol exposed hepatocytes, liver slices and in livers from ethanol-fed rats. We have since correlated alcohol-induced microtubule acetylation with known alcohol-induced defects in protein sorting including basolateral secretion, basolateral-to-apical transcytosis and the nuclear translocation of STATs. More recently, we have established that in hepatic cells treated with alcohol and oleic acid (to mimic the Western diet and fatty liver) that lipid droplets are virtually stationary. By overexpressing the tubulin specific acetyltransferase, αTAT1, we further determined that impaired droplet motility was explained by microtubule hyper acetylation. Our attention is now focused on known alcohol-induced defects in mitochondrial structure and dynamics in the absence or presence of the Western diet in the context of acetylated microtubules. From live cell imaging of vital dye-labeled mitochondria, we determined that mitochondria were virtually stationary in alcohol-treated cells and that they appeared much shorter. This result is consistent with recent reports that determined that microtubule acetylation enhances mitochondrial fission. Surprisingly, mitochondria in alcohol-treated cells in the presence of oleic acid, were longer than in control implying decreased fission. This might be explained by the finding that mitochondria form tight associations with lipid droplets (referred to as peridroplet mitochondria). When viewed by EM, we observed abundant mitochondria-droplet associations consistent with this hypothesis. We are now overexpressing αTAT1 in control and oleic acid treated-cells to further discriminate the role of microtubule acetylation in mitochondria-droplet contacts and dynamics and are examining droplet-mitochondria contact sites in control cells compared to ethanol-treated cells in absence or presence of lipid droplets to better understand mitochondria dysfunction in alcoholic liver disease.

P2279/B530

Near-infrared Light Exposure Is Associated with Increases in Mitochondrial Activity.

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Photobiomodulation (PBM) is the use of red or near-infrared (NIR) light at low power densities, to produce beneficial physiological effects on cells or tissues. This exposure to low-level NIR light has been shown to reduce pain, inflammation, and promote wound healing. It has also been shown to have several beneficial neurological effects, such as improvements on major depressive disorder and Alzheimer's Disease, as well as traumatic brain injury and stroke. Cytochrome c oxidase (CCO), the last enzyme in the electron transport chain (ETC), is believed to be the major chromophore for NIR light. The exact mechanism by which NIR mediates PBM is still not clearly understood. As CCO is a member of the ETC, the goal of this study was to characterize the effect of NIR light exposures shown to mediate PBM on mitochondrial activity. Exposure to 2.88 J/cm2 of NIR light (673 nm), 24-hours prior, induces resistance to cell death in retinal pigmented epithelial cells exposed to a 1 sec pulse of 2 μm laser radiation. Thus, we focus on this exposure in this study. The fluorescent molecule tetramethylrhodamine, ethyl ester (TMRE) was used to assay mitochondrial activity. TMRE is a cell permeant, positively-charged, red-orange dye that readily accumulates in active mitochondria due to their relative negative charge. Depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE. Results from our analysis suggest that exposure to 2.88 J/cm2 of NIR light (673 nm) results in increased mitochondrial activity.

P2280/B531

Branched-Chain Amino Acids Control Mitochondrial Metabolite Carriers Via the Mitochondrial-Derived Compartment Pathway.

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Mitochondria are double membrane-bound organelles that fulfill central tasks in cellular metabolism. Metabolite transport into and out of mitochondria is mediated by carrier proteins of the SLC25A family embedded in the inner mitochondrial membrane. Despite being critical regulatory points in cellular metabolism, it remains unclear how mitochondrial carriers are modulated in response to changes in the intracellular nutrient status. We recently identified a new subcellular structure that forms from yeast mitochondria, called the Mitochondrial-Derived Compartment (MDC). In aging yeast cells, MDCs are formed in response to defects in lysosomal amino acid storage and target select mitochondrial proteins for degradation. However, the function of the MDC pathway and the underlying signal activating MDC formation are not known. Here we present new evidence that MDCs are conserved from yeast to mammals and control the levels of metabolite carriers on mitochondria in response to intracellular amino acid elevation. Using super-resolution live-cell imaging, we demonstrate that MDCs are large (1um), organelle-like structures that selectively sort and remove metabolite carriers and their associated import receptor Tom70 from yeast and mammalian mitochondria. We show that MDCs form from mitochondria in response to intracellular amino acid elevation with branched-chain amino acids (BCAAs) being the most potent inducers of MDC formation. Our data indicates that BCAA catabolism by the branched-chain amino acid transaminases *BAT1* and *BAT2* is a major driver of MDC formation in yeast and that downstream products of BCAA degradation directly activate MDC-dependent remodeling of mitochondria. By contrast, the MDC pathway is unresponsive to other common mitochondrial stressors,

including ROS generators, inhibitors of the mitochondrial respiratory chain and membrane potential uncouplers. Since MDCs sort and remove metabolite carriers from mitochondria, we propose that the MDC pathway provides cells with a mechanism to fine-tune mitochondrial metabolite transport in response to BCAA elevation by controlling the level of select SLC25A carriers on mitochondria. In support of this idea, our preliminary data indicate that overexpression of mitochondrial carriers directly activates MDC formation in yeast, whereas deletion of the carrier receptor Tom70 reduces MDC formation. Our current experiments are focused on testing how MDC formation affects cellular and mitochondrial metabolism and identifying the machinery and metabolic signaling mechanisms that control selective sorting and removal of nutrient carriers from mitochondria via the MDC pathway.

P2281/B532

Characterization of Mitochondrial Metabolic Oscillations in Live Rodents.

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Mitochondria are specialized cellular compartments that function in energy production and calcium homeostasis. Although these organelles have been primarily investigated in cell cultures, very little is known about their morphology, function, and dynamic properties in live multicellular organisms. To address this issue, we used Intravital Subcellular Microscopy (ISMic), an imaging approach that enables the visualization of biological processes in live animals. In rat salivary glands, we previously discovered that: 1) mitochondrial metabolic activity exhibits rapid and periodic oscillations under basal conditions, and 2) mitochondrial oscillations are synchronized throughout the salivary epithelium via gap junctions. Importantly, dysfunctional mitochondrial oscillations are linked to many metabolic diseases e.g. obesity, cardiovascular disease, developmental defects and cancer, thus prompting us to ask: i) what is the physiological role of mitochondrial oscillations and ii) how oscillations are orchestrated at the whole organismal level. Here, we extended our characterization in rat and mouse tissues under both physiological and pathological conditions. Under basal conditions, we found that mitochondrial oscillations occur in all tissues tested, with substantial differences in their period, amplitude, and coordination, that most likely reflect the metabolic status of the tissues analyzed. Additionally, we identified that the metabolic oscillatory pattern is significantly altered in aged mice and during tumor progression in a mouse model for head and neck squamous cell carcinoma. In conclusion, we provided for the first time a detailed quantitative analysis of the characteristics of the metabolic oscillations under basal conditions, which can be served as a baseline to study these processes during pathological states.

P2282/B533

Mitochondria-localized β-actin Is Essential for Priming Antiviral Innate Immune Signaling by Regulating Irf3 Protein Stability.

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Antiviral innate immunity acts as a defensive barrier to viral infection in both immune and non-immune cells. The viral nucleic acids present in the cytoplasm after viral replication can activate sensors such as RIG-I-like receptors RIG-I and MDA5, or cytosolic DNA sensors cGAS to initiate signaling transduction.

Activation of this signaling cascade leads to phosphorylation of protein kinase TBK1, which in turn phosphorylates and activates transcription factor IRF3 to up-regulate type-I interferons α and β and suppression of viral replication. IRF3 is essential for sustained antiviral responses and alteration of mitochondrial function can affect antiviral immune signaling. However, whether mitochondrial defects affect IRF3 protein stability remains unclear. We recently reported the presence of a mitochondrial β-actin pool that regulates the mitochondrial genome. Loss of mitochondrial β-actin leads to decreased mitochondrial DNA transcription and impairs mitochondrial membrane potential (MMP). Mitochondrial β-actin is, therefore, essential for mitochondrial quality. In the present study, we discovered a novel function for the mitochondrial β-actin pool in the maintenance of IRF3 stability at the protein level. Using a genomics approach, we found that the lack of β-actin not only causes systematic down-regulation of genes involved in antiviral innate immune pathways, but also impairs the induction of antiviral response genes upon viral mimic stimulation. This seems to result from the instability of IRF3 in β-actin knockout mouse embryonic fibroblasts (KO MEFs). We show that reintroduction of β-actin in mitochondria of KO MEFs can rescue the stability of IRF3 and the induction of antiviral genes. We propose that mitochondria-targeted β-actin is essential for the IRF3 protein stability and effective activation of antiviral immune responses by controlling mitochondrial quality. Future studies can focus on the mechanistic basis of the link between β-actin-dependent mitochondrial quality and IRF3 stability.

P2283/B534

Mitochondrial Nucleoids Self-assemble Via Phase Separation.

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Mitochondria contain an autonomous and spatially segregated genome. The mitochondrial DNA (mtDNA) is coated by architectural proteins to form physically discrete mitochondrial nucleoids, which are non-membrane bound nucleoprotein complexes of uniform size. Beyond the direct binding of protein to mtDNA, the physical mechanisms that govern how mt-nucleoids assemble, how they are maintained, and how they contribute to function remain unclear. Here, we provide direct evidence that mt-nucleoids arise from phase separation of nucleic acids and proteins. We show that the main mtDNA packaging protein mitochondrial transcription factor a (TFAM) readily phase separates into viscoelastic droplets in vitro. Mutation analysis of TFAM reveals that multivalent interactions, intrinsically disordered domains, and charged residues contribute to the observed phase behavior. Addition of mtDNA promotes the formation of multiphase structures with gel-like properties. With regards to in vivo properties of nucleoids, pharmacological disruption with known mtDNA intercalators induces liquid-like fusion events between individual mt-nucleoids and global coarsening within the mitochondrial network. In support of a link to aging, which is associated with increased reactive oxygen species production and mitochondrial dysfunction, we find that cells derived from patients with the premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS) have an increased number of damaged mitochondria with accumulation of mt-nucleoid markers. The atypical organization and enlarged structures of mt-nucleoids in the damaged mitochondria in HGPS cells resemble those generated in vitro and are associated with impaired mitochondrial function. Together, these results suggest that mt-nucleoids represent phase separated droplets of protein and mtDNA in vivo. The phase behavior of mt-nucleoids represents a novel framework for understanding their assembly, maintenance, and organization, with potential implications for normal aging.

P2284/B535

Identifying Regulatory Genes of Mitochondrial DNA Using High-throughput Imaging an alysis.

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Mitochondria are semi-autonomous organelles containing their own genetic material. Mitochondrial DNA (mtDNA) encodes for essential proteins required for energy production via oxidative phosphorylation and is packaged into protein-DNA structures, termed nucleoids. Mutations in mtDNA and altered mtDNA copy number are linked to human diseases, including metabolic disorders. We conducted a genome-wide high-throughput high-content imaging screen in budding yeast to investigate how nucleoid structure and copy number are maintained in cells. We generated and imaged cells expressing fluorescent cytosolic, mitochondrial, and nucleoid markers using the yeast knockout library of approximately 6,000 mutants. Using CellProfiler and an unsupervised machine learning outlier detection program, we identified genes that when deleted, caused nucleoid phenotypes that significantly deviated from wild-type with a high penetrance. To further classify these phenotypes and to find additional mutations that fit specific classes of morphological profiles, we are using a supervised machine learning program trained for each phenotypic category on approximately 400 manually labeled individual cells. Ultimately, we aim to elucidate the mechanisms of mtDNA maintenance by identifying and characterizing the nuclear genes associated with mtDNA regulation.

P2285/B536

Mitochondrial Transplantation Regenerates Tendinopathy: in Vivo Study.

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Tendinopathy refers to painful conditions associated with tendons. Its main clinical symptom is activity-related pain. As its pathophysiology, inflammatory mechanisms has been highlighted. Several cytokines are activated to modulate inflammation and mitochondrial dynamics required for tendon homeostasis are also impaired. Mitochondria are known to be associated with tendon regeneration. However, there are scanty references of the action of mitochondria in tendon. Previously, we showed that direct mitochondrial transfer into muscle cells improved their metabolic function. Out hypothesis is that direct mitochondrial transplantation will also be effective in animal model of tendinopathy. First, we established a rat tendinopathy model after injection of collagenase I into Achilles tendons for 2 weeks. Isolated mitochondria were injected locally into Achilles tendons. At 2 and 4 weeks after collagenase injection, rats were sacrificed for tendon harvesting. Then, tendon tissues were analyzed to measure the change of inflammatory signaling pathway (Nuclear Factor-kβ (NF-kβ)) and tenocyte-specific markers (tenascin C (TNC), and matrix metalloproteinase 1 (MMP1)) by western blotting. We confirmed that isolated mitochondria were transplanted efficiently in tendon using confocal analysis. As a result, transplanted mitochondria (Red) were detected in extracted tendon and merged with recipient tendons (Green). Also, up-regulated p65 subunit of NF-kβ level was suppressed to normal conditions. TNC and MMP1 were increased 2.5 fold in the collagenase-injected group and inhibited in the mitochondrial transplanted group markedly. In conclusion, mitochondrial transplantation provides novel protection for tendinopathy-induced inflammatory environments. These results infer that the mitochondrial transplantation would be one of therapeutic options for tendinopathy.

Receptors, Transporters and Channels

P2286/B537

Gating Mechanism of the Extracellular Entry to the Lipid Pathway in a TMEM16 Scramblase.

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Members of the TMEM16/ANO family of membrane proteins are Ca^{2+}-activated phospholipid scramblases and/or Cl^- channels. A membrane-exposed hydrophilic groove in these proteins serves as a shared translocation pathway for ions and lipids. However, the mechanism by which lipids gain access to and permeate through the groove remains poorly understood. Here, we combined quantitative scrambling assays and molecular dynamic simulations to identify the key steps regulating lipid movement through the groove. Lipid scrambling is limited by two constrictions defined by evolutionarily conserved charged and polar residues, one extracellular and the other near the membrane mid-point. The region between these constrictions is inaccessible to lipids and water molecules, suggesting that the groove is in a non-conductive conformation. A sequence of lipid-triggered reorganizations of interactions between these residues and the permeating lipids propagates from the extracellular entryway to the central constriction, allowing the groove to open and coordinate the headgroups of transiting lipids

P2287/B538

Functional an alyses of the Udp-galactose Transporter Solute Carrier Family 35 Member A2 (slc35a2) Using the Binding of Bacterial Shiga Toxins as a Novel Activity Assay.

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SLC35A2 transports UDP-galactose from the cytosol to the lumen of the Golgi apparatus and endoplasmic reticulum for glycosylation. Mutations in SLC35A2 induce a congenital disorder of glycosylation. Despite the biomedical relevance, mechanisms of transport via SLC35A2 and the impact of disease-associated mutations on activity are unclear. To address these issues, we generated a predicted structure of SLC35A2 and assayed for the effects of a set of structural and disease-associated mutations. Activity assays were performed using a rescue approach in ΔSLC35A2 cells and took advantage of the fact that SLC35A2 is required for expression of the glycosphingolipid globotriaosylceramide (Gb3), the cell surface receptor for Shiga toxin 1 (STx1) and 2 (STx2). The N- and C-terminal cytoplasmic loops of SLC35A2 were dispensable for activity, but two critical glycine (Gly-202 and Gly-214), glutamate (E-75 and E-221), and lysine (Lys-78 and Lys-297) residues in transmembrane segments were required. Residues corresponding to Gly-202 and Gly-214 in the related transporter SLC35A1 are supposed to form a molecular notch to retain α-helices interaction when alternating between the Golgi- and cytoplasmic-facing states during transport. E-75 and Lys-78 form an EXXK motif that is conserved across SLC35A subfamily members. Our results suggest that a similar transporting mechanism may be involved in all SLC35A subfamily members. Among the disease-associated mutations tested, SLC35A2 function was completely inhibited by F65L, Y130C, S213F, and G282R, providing a straightforward mechanism of disease. Interestingly, some of the disease-associated mutations (V331I, N235Q, V258M, and Y267C) did not affect SLC35A2 function, suggesting that complexities beyond the loss of transporter activity may underlie disease due to these mutations. Overall, our results provide new insights into the mechanisms

of transport of SLC35A2 and improve understanding of the relationship between SLC35A2 mutations and disease.

P2288/B539

Characterization of a Novel Cancer-associated Splice Variant Isoform of the Nucleotide Sugar Transporter SLC35A3.

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Adaptation of the cellular secretory pathway to meet the metabolic and environmental demands of cancer cells is critical for oncogenesis and tumor growth. N-linked glycosylation represents a key regulatory step in the processing of membrane-bound and secreted proteins and is mediated by members of the SLC35 family of nucleotide sugar transporters. We report here the first characterization of a novel cancer-associated splice variant of the SLC35A3 transporter, which facilitates the transport of UDP-N-acetylglucosamine from the cytoplasm into the Golgi. The aim of the study was to compare the molecular and functional properties of the canonical SLC35A3 protein isoform 1 (NP_036375) with the SLC35A3 splice variant isoform 2 (NP_001258614). We examined SLC35A3 expression, localization, and function in human hepatocellular carcinoma (HCC), the most common form of primary liver cancer. We performed RNAseq analysis on paired surgical specimens from adjacent non-tumor and tumor tissue from HCC patients. Changes in mRNA isoform expression were validated via qPCR using sequence-specific primers. We generated expression plasmids encoding each isoform and examined protein expression, localization, and function in HCC cells. Glycosylation changes were monitored by click-chemistry approaches and lectin immunoblotting. Compared to normal human liver, HCC cell lines (HuH7, Hep3B, MHCC97, SNU423, SNU449, and PLC/PRF/5) expressed 3-15 fold less mRNA encoding SLC35A3 isoform 1, and 5-25 fold more mRNA encoding SLC35A3 isoform 2. Similarly, in primary HCC tumors, isoform 2 was selectively upregulated over isoform 1 ($p=0.00659$). Immunoblot analysis of lysates from primary HCC tumors revealed abundant expression of protein consistent with the predicted size of SLC35A3 isoform 2. Both isoforms showed strong co-localization with the Golgi marker GM130 when expressed in HCC cells. Expression of SLC35A3 isoform 2 did not alter the addition of terminal sialic acid moiety in glycoproteins. However, lectin binding assay using dolichos biflorus agglutinin (DBA) lectin revealed an increased in glycosylated proteins enriched in N-Acetylgalactosamine (GalNAc) in cells transfected with SLC35A3 isoform 2, suggesting a shift in GalNAc transport into the Golgi. Furthermore, we observed a reduction in glycosylated protein levels using griffonia simplicifolia lectin (GSL), with specificity for D-galactose, in cells transfected with SLC35A3 isoform 2. In conclusion, we show that HCC is associated with increased expression of SLC35A3 isoform 2. This possibly leads to a shift in GalNAc- and D-galactose-associated transport into the Golgi, and subsequent altered glycosylation of proteins in HCC. Future studies will address detailed functional mechanisms of SLC35A3 isoform 2 in HCC.

P2289/B540

Identifying the Amino Acid Residues Responsible for a Gating Function in Pdr5, a Major Yeast Multidrug Efflux Pump.

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The yeast ABC asymmetric transporter Pdr5 is the founding member of a subfamily of fungal transporters that cause broad drug resistance when they are overexpressed. Pdr5 acts as a one-way

gate or molecular diode to prevent reentry of substrates once they are expelled. The first evidence for a molecular gate or diode mechanism was inferred from the behavior of a Ser 1368 Ala mutation in Pdr5 This mutant exhibited significant drug reflux during transport, but otherwise had no observable biochemical deficiencies. Alignments of Pdr5 with other Pdr subfamilies revealed highly conserved residues adjacent to Ser-1368 in transmembrane-helix 11 and extracellular loop 6 which are found in the carboxyl-terminus half of the transporter. A similar set of residues in the amino terminal portion was also conserved. An important question is whether the molecular diode is made up of residues from both halves of pdr5. Mutants in these residues were constructed and they have tested for their relative drug resistance and their transport and gating capability. Our data indicate that two mutants, S678Y (amino terminal half), and F1369A, behave much like the Ser1368ala mutant. Several other mutants have phenotypes suggesting that they have a gate that is slow to open and close.

P2290/B541

Nonsynonymous Mutations in the Yeast Multidrug Transporter Pdr5 Can Increase Expression by Increasing Mrna Half-life.

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Multidrug resistance is a huge clinical problem for the cancer chemotherapeutic and treatment of fungal infection. Enhanced expression of the ABC transporter protein is one of the leading mechanistic explanation of this broad resistance. PDR5, a major efflux ABC transporter protein, mediates broad-spectrum resistance in *Saccharomyces cerevisiae*. Previous work established that mutations in transcription factors lead to Pdr5 overexpression. We report the novel observation that a series of nonsynonymous mutations in an unconserved stretch of amino acids found in the yeast multidrug efflux pump Pdr5 increases expression, thus enhancing multidrug resistance. Cycloheximide chase experiments ruled out the possibility that the increased steady-state level of PDR5 was caused by increased protein stability. Quantitative-RT PCR experiments demonstrated that the mutants had levels of Pdr5 transcript that were two to three times as high as in the isogenic wild-type strain. Further experiments with metabolic labeling of mRNA with 4-thiouracil followed by uracil chasing showed that the half-life of Pdr5 transcripts was specifically increased in these mutants. Our data demonstrate that the nucleotides encoding unconserved amino acids may be used to regulate expression.

P2291/B542

The Molecular Mechanism Underlying Growth Inhibition of *Saccharomyces Cerevisiae* by Supplementing Lysine to the Medium Containing Proline as Sole Nitrogen Source.

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The budding yeast *Saccharomyces cerevisiae* can grow on the medium containing proline as sole nitrogen source (SD+Pro). It has been shown that the addition of lysine (Lys) to the SD+Pro inhibits the growth. We found that the deletion of *AVT4* encoding a transporter, which exports neutral and basic amino acids from vacuoles, further suppressed the growth in SD+Pro+Lys medium. To dissect these effect of lysine, we cultured wild-type cells in SD+Pro or SD+Pro+Lys and measured the amino acid contents of the whole cell, vacuoles, and culture medium. When compared with cells cultured in

SD+Pro, cells cultured in SD+Pro+Lys contained much less amount of proline, whereas they contained the a large amount of lysine. In *avt4*Δ cells, such high and low contents of lysine and proline, respectively, were sustained for longer time than in wild-type cells. Consistently, proline remained in the medium for longer time in the case of *avt4*Δ cells. A major part of cellular lysine in both wild-type and *avt4*Δ cells cultured in SD+Pro+Lys was located in vacuoles. We found that Put4, a plasma membrane transporter for the proline uptake, was translocated into the vacuolar lumen in the presence of lysine. In addition, the expression of Put4-20, a mutant which constitutively localizes to the plasma membrane, recovered the growth of both the wild-type and *avt4*Δ cells in SD+Pro+Lys. These suggest that the growth inhibition by addition of lysine is due to the reduction of cellular proline uptake, and vacuolar lysine accumulation may block the targeting of Put4 to the plasma membrane. Then we examined the involvement of TORC1 activity, which regulates the expression and subcellular localization of Put4. The deletion of *GTR1 and GTR2*, which blocks TORC1 activation, resulted in the growth recovery in SD+Pro+Lys. We found that the addition of lysine extended the phosphorylation of Sch9, a substrate of TORC1. These suggests that lysine has some effect to activate TORC1 though it is not utilized as a nitrogen source.

P2292/B543

Vacuolar Amino Acid Transport Is Regulated by the GATA Transcription Factors.

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Vacuoles in the budding yeast *Saccharomyces cerevisiae* actively uptake amino acids and accumulate more than 50% of cellular amino acids in nutrient-rich condition, whereas they release the accumulated amino acids with newly generated ones by autophagy under nitrogen starvation condition. Such conversion of amino acid flux across the vacuolar membrane suggests that the activities of vacuolar amino acid transporters are regulated in a manner dependent on nutritional condition. The AVT transporter family includes vacuolar amino acid transporters such as Avt1, Avt3, Avt4, Avt6 and Avt7. Avt4 is a vacuolar exporter for neutral and basic amino acids. Previous microarray analysis suggested that its expression increases under nitrogen starvation condition and is regulated by the GATA transcription factors, Gln3 and Gat1, which are responsible for the transcription of genes related to the nitrogen metabolism to adapt cells to the change in the availability and quality of nitrogen source. Our real-time PCR analysis supported the previous microarray results. Mutations in the putative GATA binding sites in the *AVT4* promoter reduced the expression of *AVT4*. In addition, Gat1-myc^{13} specifically precipitated the *AVT4* promoter fragment containing these GATA binding sites in the chromatin immunoprecipitation assay. These results suggest that the GATA transcription factors directly drive the *AVT4* transcription. Confirming the microarray results, real-time PCR analysis showed that *AVT1* and *AVT7* expressions were also down-regulated by the deletion of the GATA transcription factors. In addition, we newly found that *AVT6* is also regulated by the GATA transcription factors. Taken together, these results suggest that the GATA transcription factors broadly regulate the vacuolar amino acid transport system. Considering the physiological roles of GATA transcription factors, our results in this study strongly suggest that the vacuolar compartmentalization of amino acids by their transport across the vacuolar membrane is incorporated to the cellular nitrogen metabolism.

P2293/B544

The Role of Basigin as an Immune Mediator in the CNS.

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Chronic inflammation is a hallmark of many neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (MS). Although the central nervous system (CNS) can stave peripheral pathogens from crossing the blood-brain barrier (BBB) through a network of continuous nonfenestrated endothelia, astrocytes, and pericytes, prolonged exposure to a pathogen can comprise this barrier. An increase of IL-6 can stimulate vascular cell adhesion molecule 1 (VCAM-1), leading to increased permeability of the BBB, effectively allowing the infiltration of T-cells and cytokines, such as IL-6. T-cells, as well as macrophages, are lined with surface receptors that detect pathogens and elicit further immune responses, particularly toll-like receptor 4 (TLR4). TLR4 has been demonstrated to detect lipopolysaccharide (LPS), found on the outer membrane of Gram-negative bacteria. Recently, a member of the immunoglobulin superfamily (IgSF), Basigin, has been shown to interact with TLR4. Studies have also indicated that Basigin is expressed on endothelial cells and can spur angiogenesis. The present study aims to address the expression pattern of Basigin and TLR4 in brain tissue stimulated with LPS for a variation of time to mirror acute and chronic inflammation, as well as different life stages to determine whether the expression pattern is dynamic. Isolated brain tissue and neural retina from mice at postnatal day 7 and 30 were incubated in DMEM ± LPS for 3, 6, 12, or 24hrs. Total RNA and protein were purified from the isolated tissue and used in quantitative reverse transcription PCR (qRT-PCR) and direct enzyme-linked immunosorbent assay (ELISA). The results of the study suggest expression of Basigin is elevated after 24 hours of incubation in LPS relative to control in the 30-day old animals, but not in the 7-day old animals. This elevation in expression was not observed at other incubation periods. Expression of TLR4 did not change between age groups or incubation periods. The data suggest that expression of Basigin, but not TLR4, changes from neonate to adolescent age. This may leave the neonatal CNS more susceptible to an inflammatory response.

P2294/B545

The Ig0 Domain of Basigin Variant-1 Stimulates Il-6 Expression in Raw 264.7 Monocytes.

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There are two main protein products of the Basigin gene. One protein, known as Basigin-variant-1 is expressed in the neural retina, specifically by the photoreceptor neurons. The other protein, known as Basigin-variant-2 is expressed throughout the body, including monocytes, as well as Müller glial cells and the retinal pigmented epithelium of the eye. A study by this laboratory indicates that the two Basigin gene products interact via their extracellular domains. A different study by another research group indicates that the Ig0 domain of Basigin-variant-1 can elicit an immune response in several cell lines. The purpose of the present study was to determine if the region of the Ig0 domain of Basigin-variant-1 thought to interact with Basigin-variant-2 is the same region that elicits an immune response. Recombinant versions of the Basigin-variant-1 Ig0 domain were incubated with mouse monocytic RAW 264.7 cells. After 24 hours, the cell culture medium was collected and assayed for the expression of the pro-inflammatory cytokine interleukin-6 (IL-6) via an ELISA. Cells treated with a control protein generated from the expression vector used to make the Basigin-variant-1 recombinant proteins served as the control. The data indicate that the region of the Ig0 domain used to interact with Basigin-variant-2 does indeed stimulate production of a significantly greater amount of IL-6 than the control protein.

The data suggest that Basigin-variant-1 interacts with Basigin-variant-2 to stimulate a proinflammatory response in monocytes. This study is important for a better understanding of immune response aspects of diabetic retinopathy and other diseases in which the retinal pigmented epithelium is compromised.

P2295/B546

Ampa and Nmda Receptor Alterations Mediate Enhanced Escalation of Methamphetamine Self-administration.

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Objectives: Methamphetamine addiction is often studied using rodent self-administration models whereby animals associate lever presses with intravenous drug infusions. Some investigators have used a pre-injection of the drug of interest before introducing rodents to the self-administration apparatus. This experimental approach usually causes steeper escalation of drug taking behaviors. The present experiment was aimed at identifying potential changes in the hippocampi of rats that had undergone a similar procedure using methamphetamine as the self-administered drug. **Methods:** Rats were first injected with either saline or methamphetamine intraperitoneally. Two weeks later, the animals underwent surgery for catheter placement. During self-administration, pressing a lever resulted in intravenous injections of methamphetamine. Rats also underwent tests for relapse to drug taking one day and one month after withdrawal from drug self-administration. We then used PCR and Western Blotting to measure potential changes in mRNA and protein expression levels of glutamate receptors. **Results:** Rats that received the methamphetamine injection before drug self-administration escalated drug intake more than the animals that received saline pre-injection. In the hippocampus, these rats showed decreased levels of *GluA2*, *GluA3*, and *GluN2c* mRNA levels in comparison to other animals. In addition, GLUA1 protein levels were decreased in the methamphetamine-preinjected rats. **Conclusion**. These observations suggest that decreased expression of hippocampal AMPA receptors may participate in escalation of methamphetamine intake. Identification of molecular mechanisms involved in drug taking behaviors may facilitate the development of better therapeutic anti-addiction interventions in the future.

P2296/B547

Putative Yeast Polycystin Channel Pkd2p Localizes to the Cleavage Furrow and Drives Cell Separation during Cytokinesis.

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Human polycystin PKD2 is a non-selective transient potential receptor (TRP) channel. It co-assembles with another polycystin PKD1 into a tetrameric complex that is essential for many developmental processes. Loss of function mutations of either polycystins lead to Autosomal Polycystic Kidney Disease (ADPKD), one of the most common human renal disorders. Nevertheless, the cellular function of polycystins remains unclear. *Here* w*e showed that the fission yeast homologue of PKD2 supprisingly localizes to the cleavage furrow and modulates multiple steps of cytokinesis.* Like its human homologue, fission yeast *pkd2* is an essential gene. Pkd2p localizes to the plasma membrane during interphase but it is recruited to the cleavage furrow by the actomyosin contractile ring during cytokinesis. Its molecular number at the furrow peaks at the end of the ring constriction. Pkd2p is essential for maintaining the cell integrity during interphase. The *pkd2* mutant cells often deflate temporarily, causing a arrest of the

cytoskeletal turnover and the subsequent stress response. More interestingly, Pkd2p regulates both the contractile ring closure and the cell separation during cytokinesis. The contractile ring constricts more rapidly in the *pkd2* mutant than the wild-type cells, a defect rescued by the addition of an osmotic stabilizer sorbitol. In contrast, separation of the mutant cells is slower and often incomplete. These cytokinesis defects suggest that Pkd2p regulates turgor pressure at the cell division plane as a part of intracellular osmotic homeostasis. Lastly, a study of genetic interactions between the *pkd2* mutant and many other cytokinesis mutants found that Pkd2p act antoganostically against the Septation Initiation Network (SIN) pathway during cytokinesis. In conclusion, we discovered that the fission yeast homologue of polycystin Pkd2p is a novel regulator of cytokinesis through mediating intracellular osmotic homeostasis.

P2297/B548

Characterization of Protein Signalosome Using Super-Resolution Microscopy.

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Molecules are often organized in cellular space packed within organelles which are surrounded by lipid bilayer such as endoplasmic reticulum or Golgi apparatus. However, a variety of molecules, such as proteins and Ribonucleic Acids (RNA), have been found to form condensates in the cellular compartments without any defined boundaries. Similarly, membrane bound signaling molecules have also been observed to form aggregates similar to the condensates, also named as clusters. However, the biological or structural benefit of the formation of these clusters and how these molecules are spatially distributed within these structures are not fully understood. To understand the dynamics of the molecules in the cluster and to contribute to the theoretical and experimental knowledge of the condensates, we studied the cluster formation of Epidermal Growth Factor Receptors (EGFR). EGFR is a well-studied Receptor Tyrosine Kinases (RTK) which forms dimers after the stimulation by its ligand Epidermal Growth Factor (EGF), initiating signaling cascade by binding of the Src Homology 2 (SH2) domain containing molecules. EGFR is also implicated in several forms of cancer and is being used as a chemotherapeutic target. For this purpose, EGFR clusters were imaged using Super-Resolution Imaging technique called Stochastic Optical Reconstruction Microscopy (STORM) Imaging. For the immunochemical staining, a Halo tagged EGFR nanobody was used which gives the opportunity to stoichiometrically label total EGFR receptors. We observed that the number of clusters, as well as their size, was increased as a response to the ligand stimulation. To further analyze the spatial distribution of phosphorylated receptor molecules within the cluster, engineered Halo tagged Growth factor receptor-bound protein 2 (Grb2) probes, which bind specifically to activated SH2 binding sites on RTKs, were used for immunostaining. Our results show that Halo tagged Grb2 probes have a higher binding affinity towards the phosphorylated receptors. Moreover, by further modeling these clusters, we found that the phosphorylated molecules tend to concentrate within the core of the clusters, indicating Phosphatase activity on the peripheries of the cluster. Here we propose that the application of Halo tagged probes/nanobodies in STORM microscopy can greatly enhance the effectivity of Super-Resolution Imaging technique and help us model the clusters or other cellular structures.

P2298/B549

F508del-CFTR Forms a Complex with NHERF2 and LPA_2 at the Plasma Membrane of Airway Epithelial Cells: Its Relevance in the Pathogenic Process of Cystic Fibrosis.

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BACKGROUND: Cystic fibrosis (CF) is a life-shortening inherited disease caused by the absence or dysfunction of the CF transmembrane conductance regulator (CFTR) channel activity. Previously, we demonstrated that wild type-(WT-)CFTR forms a macromolecular complex with lysophosphatidic acid receptor 2 (LPA_2) and Na^+/H^+ exchanger regulatory factor-2 (NHERF2) at the plasma membrane of airway and intestinal epithelial cells, and that this complex regulates the LPA-induced inhibition of CFTR channel function and fluid secretion. The goals of this study were to investigate if the rescued F508del-CFTR complexes with NHERF2 and LPA_2, and if this complex plays a role in the pathogenic process of CF. **METHODS:** Study models: human CF bronchial epithelial cells, human tracheal and bronchial epithelial tissues, CF mice (*F508del*$^{-/-}$) *and* WT mice. Techniques: Western blotting, immunofluorescence imaging, proximity ligation assay, Ussing chamber, organoids fluid secretion assay, etc. **RESULTS:** (1) LPA_2 expression was elevated in tracheal and bronchial epithelial tissues from CF patients and in CF bronchial epithelial cells. (2) When rescued to the plasma membrane, F508del-CFTR complexes with NHERF2 and LPA_2 in human CF airway epithelial cells. By forming such a complex, the LPA_2-mediated signaling exerts an inhibitory effect on the channel function of F508del-CFTR. Disruption of this complex by using CO-068 potentiated the channel function of F508del-CFTR. (3) F508del-NHERF2-LPA_2 complex regulates IL-8 secretion in CF bronchial epithelial cells. Rescue of F508del-CFTR by using VX-809 slightly decreased the basal level of secreted IL-8 and significantly decreased the IL-1β-stimulated IL-8 secretion (11%). Inhibition of LPA_2 function by using a specific inhibitor Beck35 significantly decreased the basal level (44%) and IL-1β-stimulated IL-8 (~39%) secretion. CO-068 also attenuated the LPA-induced IL-8 secretion. (4) LPA_2 is expressed in primary neutrophils isolated from human blood. **CONCLUSIONS:** CFTR-NHERF2-LPA_2 complex plays a critical role in the pathogenic process of CF: in addition to regulating the CFTR-mediated airway fluid homeostasis, it also modulates IL-8 secretion from airway epithelial cells, which could contribute to the recruitment and excessive infiltration of neutrophils and the subsequent excessive lung inflammation. An tagonism of LPA_2 has a potential to augment CFTR function to improve the mucociliary clearance process, and to inhibit the release of IL-8 and other pro-inflammatory cytokines and chemokines, thereby attenuating the excessive CF lung inflammation. **Acknowledgement**: Supported by NIH grant (R01 HL123535 to W.Z.)

P2299/B550

Transferrin Receptor 2 Requires Both Its Cytoplasmic Domain and the Ability to Bind Tf to Regulate Iron Homeostasis.

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Transferrin receptor 2 (TfR2) is an integral membrane protein with a N-terminal cytoplasmic domain (CD), a transmembrane domain, and a large ectodomain responsible for its dimerization and binding the serum iron-transport protein, transferrin (Tf). It is expressed predominantly in the liver and in erythroid precursors (1). The finding that deleterious mutations in TfR2 or lack of Tfr2 in mice are responsible for iron overload, led to the hypothesis that this receptor is involved in systemic iron regulation (2,3, rather than solely in Tf-mediated iron uptake. Iron homeostasis in the body is controlled by hepcidin, a small

peptide hormone secreted by the liver. Hepcidin downregulates the iron exporter ferroportin and limits the recycling of iron from macrophages and the transit of dietary iron from intestinal epithelial cell into the circulation. Under high iron conditions, hepcidin increases to limit the amount of iron entering circulation via macrophages and intestinal epithelial cells. Hereditary hemochromatosis type III is caused by a lack of TfR2, or deleterious mutations in TfR2, and results in a pathophysiology of abnormally low hepcidin levels for the amount of iron in the body. In this report, we sought to determine the domains of TfR2 necessary for regulation of hepcidin production by the liver. We found that both the CD of TfR2 and the ability of TfR2 to bind Tf are necessary for the protein to properly modulate hepcidin expression. Interestingly, the ability of TfR2 to internalize appears to be independent of its ability to upregulate hepcidin levels, implying that TfR2 is able to signal while still at the cell surface.

P2300/B551

Influence of Genipin on the Activity and Presence of Amylin in Sh-sy5y and Rin-5f Cells.

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Amylin is a fibrillogenic protein co-secreted with insulin from pancreatic beta cells. Amylin has been shown to function in metabolism through its influence on glucose homeostasis, which includes sending satiety signals to the brain, inhibiting glucagon secretion and delaying gastric emptying. In addition to amylin's direct role on metabolism it has also been proposed to have an impact on amyloid beta, a major component of the amyloid plaques found in Alzheimer's disease. Alzheimer's disease brains have been shown to have amyloid plaque formation anddecreased expression of Uncoupling Protein 2 (UCP2), which is a protein that also has a role in metabolism. Traditionally, uncoupling proteins have been known to function as mitochondrial transporter proteins that create proton leaks across the inner mitochondrial membrane resulting in the dissipation of energy in the form of heat. Knowing that UCP2 is expressed in pancreatic beta cells and has been shown to have diminished levels in Alzheimer's disease brains the focus of this study was to examine the influence of UCP2 on the presence and expression of amylin in both RIN-5F cells (a pancreatic beta cell line) and SH-SY5Y cells (a neuroblastoma cell line). Immunocytochemistry experiments and ELISA assays were conducted with and without Genipin, an inhibitor of UCP2, to examine both the presence of amylin and the activity level of amylin, respectively. Initial studies indicate that pharmacologically inhibiting UCP2 impacts amylin activity level and possibly influences the presence of amylin.

P2301/B552

Polyunsaturated Fatty Acids Inhibit the Formation of Myotubes by Activating the Free Fatty Acids Receptors 1 and 4 in C_2C_{12} Cells.

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Introduction: Polyunsaturated fatty acids (PUFAs), both omega-3 and omega-6, are commonly consumed by the world population. Normally, the intake of omega-3 has positive effects on the cardiovascular system and on the contrary, the high intake of omega-6 would have harmful effects on health. PUFAs activate free fatty acid receptors 1 (FFAR1) and FFAR4, but their effect on muscle tissue has not been described. **Objective**: to demonstrate that the presence of PUFAs (omega-3 and omega-6) induce the activation of FFAR1 and/or FFAR4, which inhibit the formation of myotubes in C2C12 cells.

Methods: C2C12 cell line was used. These cells were differentiated (10 days) in DMEM medium supplemented with 5% horse serum, in the absence or presence of alpha-linolenic acid (ALA, omega-6) or linoleic acid (LA, omega-3), and agonist or antagonist of FFARs (GW9508 and GW1100, respectively). Myogenesis markers (e.g., MyHC and MyoG) were analyzed by Western blot (WB), immunofluorescence (IF) or PCR. Also, a protein kinase associated with FFARs, as AKT, was analyzed (total and phosphorylated) by WB. **Results**: the formation of myotubes (differentiation process) was verified by the expression of MyHC and MyoG. Myotube number decreased in presence (1 to 10 days) of ALA (100 µM), LA (100 µM) or the FFAR agonist: GW9508 (50 µM). Also, the presence of ALA or LA reduce the expression of phosphorylated AKT but this reduction is not present with GW1100 (FFAR1 antagonist). Also, the presence (1 to 10 days) of LA or ALA increase the expression of FFAR4 in C2C12 cells, and this increase was blocked in presence of GW1100 (10 µM). **Conclusion**: These results provide an active role for polyunsaturated fatty acids and their receptors (FFAR1 and FFAR4) in the muscle cells differentiation. **Acknowledgment**: This work was supported by FONDECYT: 11160536 (CP), 11160739 (LC).

P2302/B553

Ca^{2+}Spikes Induced by Changes in Membrane Tension in ER Membranes Instructs the Polarized Trafficking of Cargoes in Astrocytic Processes.

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Calcium signaling with high spatial and temporal resolution in cells is known to regulate many biological processes. Nonetheless, the mechanism and functional significance of spontaneous calcium spikes found primarily in astrocytic processes is not fully understood. By developing an endoplasmic reticulum (ER)-anchored membrane tension sensor based on TSMods, we found that calcium spikes detected by GCaMP6f in astrocytic processes usually occur in regions of higher ER membrane tension. We next manipulated ER membrane tension in astrocytes by indirectly changing the lipid composition of ER membranes by an Auxin-inducible phospholipase system or by directly exerting additional force to the ER by photo-inducible, transient recruitment of kinesin. We found that calcium spikes detected by GCaMP6f increased when ER membrane tension increased, and vice versa. Co-expression of GCaMP6f with different vesicle markers/cargoes further revealed that increased local calcium spikes induced by increased ER membrane tension promoted anterograde transport of vesicles/cargoes into distal processes of astrocytes. Based on these results, we propose that increased membrane tension in theER facilitates Ca^{2+} release, which, in turn, triggers anterograde transport of vesicles into distal processes of astrocytes.

Kinases and Phosphatases 2

P2303/B555

Unraveling Mtorc1 Activity Dynamics and Its Biological Roles in Cell Cycle Progression.

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Mechanistic target of Rapamycin complex 1 (mTORC1) is a kinase that integrates diverse range of extra/intracellular inputs such as growth factors, nutrients and intracellular ATP level. It controls various downstream cellular functions such as translation, metabolism and autophagy that ultimately drive cell growth. Aberrant activation of the kinase leads serious outcomes such as cancer and diabetes. The

mechanisms how mTORC1 activates has been energetically studied. Despite this, the dynamics when mTORC1 is activated in living cells is barely understood. Due to this lack of temporal information of mTORC1 activation, it is almost unknown that how mTORC1 controls its downstream functions in a coordinated manner (or sometimes selective manner) by utilizing dynamics of its own activity. To understand the entity of mTORC1 activity dynamics and dynamic encoding of information for cellular functions with it, I aimed to visualize mTORC1 activity in living cells. For this, I stably expressed Eevee-S6K, a previously developed intramolecular FRET probe which monitors the activity of S6 kinase direct downstream of mTORC1 into several cancer cells treated with MEK inhibitor, an inhibitor of Ras-ERK MAPK pathway. mTORC1(S6K) activity in probe-expressing cells were visualized by time-lapse FRET imaging. I found that mTORC1 activity in cells sensitive to MEK inhibitor (i.e. showing growth suppression by the inhibitor) monotonously deceased by MEK inhibitor treatment. On the other hand, cells resistant to MEK inhibitor (i.e. showing prolonged cell growth under the inhibitor treatment) showed fluctuations of mTORC1 activity at single cell level. Interestingly, both sensitive cells and resistant cells showed fluctuations of the activity but they seemed to be different from the temporal activity patterns of MEK inhibitor-treated resistant cells in terms of amplitude and frequency. These results indicate the possibility that information for cell growth is encoded in dynamics of mTORC1 activity. In this presentation, I would like to talk about the mTORC1 activity dynamics in MEK inhibitor-sensitive and resistant cancer cells. Recently, to address the dynamic encoding of cell proliferation with mTORC1 activity, I have been trying to visualize mTORC1 activity and cell cycle progression simultaneously by live cell imaging utilizing Eevee-S6K and Fucci(CA), a variant of Fucci fluorescent cell cycle indicator. I will also discuss the progress of the dual monitoring.

P2304/B556

Functional Characterization of the TSC-DYRK1A Interaction.

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Functional characterization of the TSC-DYRK1A Interaction the Tuberous sclerosis complex (TSC) includes TSC1, TSC2 and the TBC1D7 subunits that together function as a principal inhibitor of the mTOR protein kinase complex 1 (mTORC1). mTORC1 is a master regulator of cell growth and proliferation that responds to signaling cues such as growth factors and nutrient availability. Proteomic studies in our lab revealed an interaction between the TSC subunits and DYRK1A, a ubiquitous protein kinase encoded by a gene located in the Down syndrome (DS) region on human chr21. In this study, we sought to validate the interaction of the TSC complex with DYRK1A and to determine its functional significance. Our analysis confirmed that DYRK1A interacts with TSC1 at the endogenous levels, and with TSC2 when overexpressed. Domain mapping of the DYRK1A-TSC interaction revealed that binding of TSC2 to DYRK1A requires TSC1. However, binding of TSC1 to DYRK1A does not require TSC2 as evidenced by the DYRK1A-TSC1 interaction observed in TSC2-null cells. Given that TSC is a major inhibitor of the mTORC1 pathway, we sought to assess the effect of DYRK1A loss on the mTORC1 activity. Interestingly, we did not observe any change in phosphorylation of mTORC1 substrates p70S6K and 4EBP1 under serum-starved conditions in the DYRK1A siRNA treated cells or in the CRISPR-Cas9 DYRK1A knockout (KO) cells. However, using FACS analysis, we noticed that human and mouse cells lacking DYRK1A appeared smaller in size compared to controls. We also observed that global protein synthesis rate was significantly reduced in DYRK1A KO cell lines using the puromycin-tagging assay. Interestingly, the decrease in global protein production was independent of the presence of TSC1. Therefore, it is possible that interaction of DYRK1A with the TSC complex could have an effect on the function of DYRK1A Kinase itself. Indeed, our

preliminary studies show that DYRK1A kinase activity towards its substrate Lin52 was decreased in the TSC1-null MEFs. Current studies are focused on confirming this effect using TSC1 knockdown human cell lines as well as the TSC1 rescue experiments in MEFs. Overall, our study introduces DYRK1A as a novel partner of the TSC complex that could mediate its non-canonical functions including mTORC1-independent regulation of cell proliferation and protein synthesis.

P2305/B557

Regulation of Phosphoinositide Turnover during Insulin Signaling.

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Insulin signaling is an evolutionarily conserved inter-cellular communication pathway in metazoans. The core components that transduce insulin signal into changes in the activity of downstream effectors have been extensively studied. While most effectors studied in this pathway are proteins, other types of biomolecules including phosphoinositides also play an important role. Phosphoinositides are phospholipids that have a critical role in orchestrating many cellular signaling cascades in spite of their very low abundance on cellular membranes. During insulin signaling, a key event is the generation and robust control of small amounts of the lipid phosphatidylinositol 3,4,5-trisphosphate (PIP_3) by Class I phosphoinositide 3-kinase (PI3K) at the plasma membrane. Dysregulation in PIP_3 levels disrupt normal growth and metabolism. Mechanistic insights into regulation of the levels and the subcellular localization of these phosphoinositide lipids are useful in understanding ways by which the insulin signaling cascade maintains its signaling fidelity while allowing for feedback regulation and signaling crosstalk. We report that in *Drosophila*, interaction between the lipid kinases - Class I PI3K and phosphatidylinositol 5 phosphate 4-kinase (PIP4K), is required to maintain normal PIP_3 levels during insulin receptor activation. Depletion of PIP4K increases the levels of PIP_3 produced in response to insulin stimulation due to enhanced Class I PI3K activity. While PIP4K localizes on multiple intracellular membranes, its plasma membrane localization was sufficient to regulate PIP_3 levels. Interestingly, the catalytic ability of PIP4K was dispensable for this regulation, highlighting a need to explore both kinase-dependent and independent functions of such enzymes. Additionally, increased cellular insulin sensitivity seen upon loss of PIP4K partially suppresses insulin resistance phenotypes in *Drosophila* larvae. PIP4Ks could thus be key regulators of receptor tyrosine kinase signals affecting processes like tumour growth in cancers, T-cell activation and metabolism in Type II diabetes. Ongoing work suggests that the interaction between these kinases is a multi-step process and entails a need to also investigate the turnover of other related phosphoinositide lipids and the activity of associated effectors during insulin stimulation.

P2306/B558

Addition of Monomer Hapten and Hapten-specific IgG Inhibits Mast Cell Activation with Different Mechanisms.

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Aggregation of IgE bound to high affinity IgE receptor (FcεRI) by multivalent antigen induces mast cell activation. Reportedly, disaggregation of aggregated FcεRI immediately terminated degranulation, and formation of co-ligated FcεRI and low affinity IgG receptor FcγRIIB blocked degranulation by inhibitory signal via SH2-containing inositol 5'-phosphatase 1 (SHIP1) phosphorylation. However, their molecular mechanisms to inhibit mast cell activation have been unclear in detail. Herein, using rat basophilic leukemia (RBL-2H3) and mouse bone marrow-derived mast cells (BMMCs), we investigated the inhibitory effects of monovalent hapten and hapten-specific IgG in FcεRI-mediated mast cell activation. Addition of multivalent hapten (TNP-OVA) induced sustained increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), which peaked at approximately 50 s after stimulation. When monovalent hapten (TNP-alanine) or hapten-specific IgG monoclonal antibody (anti-TNP IgG1) was added 50 s after TNP-OVA stimulation, $[Ca^{2+}]_i$ rapidly or gradually decreased, respectively. Further, addition of TNP-alanine after TNP-OVA stimulation completely discontinued β-hexosaminidase release, while the addition of anti-TNP IgG1 induced partial suppression of β-hexosaminidase release. Next, we investigated how the subsequent addition of TNP-alanine and anti-TNP IgG1 to mast cells activated with TNP-OVA affects the phosphorylated states of Syk and SHIP1. Interestingly, TNP-alanine addition after TNP-OVA stimulation immediately dephosphorylated the phosphorylated Syk. This dephosphorylation was transient and phosphorylation levels recovered at 5 min. The anti-TNP IgG1 addition did not induce Syk dephosphorylation and showed a similar pattern as TNP-OVA stimulation, but it induced SHIP1 phosphorylation that was not caused by TNP-alanine addition. Moreover, TNP-alanine or anti-TNP IgG1 addition after TNP-OVA stimulation inhibited TNF-α and IL-4 production through suppression of phosphorylation levels of Akt and ERK. These results suggested that the FcεRI dissociation and the co-ligation of FcεRI with FcγRIIB greatly blocked major cellular output in early phase such as degranulation and in late phase such as cytokine production, respectively.

P2307/B559

The Effect of Neuronal Growth Factors on Merlin Expression in Immortalized Schwann Cells.

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Regulation of Schwann cell growth in vitro is facilitated by heregulin, a neuron-secreted growth factor, and mitogens that activate the cyclic AMP pathway. Although the role of heregulin and forskolin has been characterized in Schwann cells, the impacts they have on the proteins merlin and ezrin have yet to be explored. Merlin, also known as "Moesin-Ezrin-Radixin-like protein" is commonly known as a Schwann cell tumor suppressor and as an A-kinase-anchoring protein (AKAP). It was hypothesized that the expression of merlin and ezrin proteins will not be altered in Schwann cells stimulated with mitogens. To determine the expression of merlin and ezrin, immortalized Schwann cell line CRL-2941 cultures were treated with control media (N2), heregulin (12.5 ng/ml), forskolin (1μM), and heregulin plus forskolin for 24 hours. Using western blotting, it was found that the expression of merlin

significantly increased with the treatment of 1μM forskolin (223.5 ± 22.14%) in comparison to heregulin treatment (147.16 ± 13.96%) but not with heregulin plus forskolin (179.35 ± 32.23%). Conversely, the expression of ezrin was highest when cells were stimulated with heregulin plus forskolin (130 ± 19.545%) in contrast to heregulin (94.97 ± 22.89%) or the forskolin treatment (97.57 ± 15.03%). To clarify the role of cAMP in the expression of ezrin, Schwann cells were treated with various concentrations of the phosphodiesterase inhibitor rolipram. Cells incubated with heregulin plus 10μM rolipram had the highest ezrin expression. Furthermore, protein levels of merlin were analyzed in Schwann cells treated with various concentrations of forskolin, at 0.5 μM, 1 μM 2 μM and 3 μM or forskolin plus heregulin for 12 and 24 hours. Although merlin exhibited a dose-dependent increase in expression with forskolin stimulation, the addition of heregulin resulted in a decrease in protein levels. Overall, cells from the 24-hour dose response exhibited higher merlin expression in comparison to the 12-hour treatment. To examine if the location of merlin is altered by the addition of heregulin and/or forskolin, immunofluorescence experiments were conducted. It was found that in cells treated with heregulin, merlin exhibited a cytoplasmic staining pattern, whereas treatment with forskolin or heregulin plus forskolin, revealed a nuclear location. This may have occurred because the addition of heregulin may have influenced the cells to proliferate, in which case merlin as a tumor suppressor is inhibited. In summary, these findings suggest that merlin is upregulated when the cAMP pathway is stimulated and downregulated when heregulin stimulates Schwann cell growth.

P2308/B560

Auto-dephosphorylation of Myosin Phosphatase Regulates Vascular Endothelial Barrier Funsction.

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Vascular endothelial cells form a selective barrier that controls the permeability of the vascular endothelium, which is critical for maintaining homeostasis. However, the mechanisms underlying the maintenance of endothelial barrier function are poorly understood. In this study, we report that a novel signaling pathway, auto-dephosphorylation of myosin phosphatase, maintains endothelial barrier function. Actomyosin contraction, which is regulated by phosphorylation of myosin light chain (MLC), increases vascular endothelial permeability. MLC phosphorylation is regulated by the balance between the enzymatic activities of myosin phosphatase and serine/threonine kinases (e.g., Rho-kinase). Myosin phosphatase, which suppresses actomyosin contractility via dephosphorylation of MLC, consists of three subunits: a myosin phosphatase targeting subunit (MYPT1), serine/threonine phosphatase catalytic subunit (PP1c), and a small 20-kDa subunit (M20). Myosin phosphatase activity is suppressed by phosphorylation of the MYPT1 subunit. Our previous in *silico* analysis predicted that myosin phosphatase dephosphorylates MYPT1, which enhances its phosphatase activity, and this auto-dephosphorylation pathway suppresses actomyosin contraction by phosphorylating MLC. Here, we provide experimental evidence supporting the predictions of our in *silico* analysis. We demonstrated that myosin phosphatase dephosphorylates MYPT1 and that its activity is promoted by this auto-dephosphorylation pathway in an in *vitro* phosphatase assay and inhibition of myosin phosphatase activity in cells. To examine the physiological function of myosin phosphatase auto-dephosphorylation, we generated a mutant that can inhibit this auto-dephosphorylation pathway. Overexpression of the mutant attenuated endothelial cell adhesion and increased permeability in human umbilical vein endothelial cells (HUVECs). Therefore, auto-dephosphorylation of myosin phosphatase regulates

endothelial adhesion and endothelial permeability. These results suggest that the auto-dephosphorylation of myosin phosphatase is essential for maintaining endothelial barrier function.

P2309/B561

Nf-κB Pathway Modulates Autophagy in Metabolic Activated Macrophages.

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Obesity is associated with the incidence of multiple co-morbidities, including metabolic syndrome and insulin resistance, the latter contributing to the development of type 2 diabetes (T2D). Underlying the development of insulin resistance by obesity is a *chronic low-grade systemic inflammation*. This meta-inflammation results from the infiltration of immune cells into the adipose tissue (AT), driving a pro-inflammatory environment. However, the molecular mechanisms responsible for the initiation and maintenance of the inflammatory response in AT are poorly understood. Therefore, the goal of this study is to investigate the intracellular players that initiate and maintain chronic meta-inflammation. We hypothesized that activation of the pro-inflammatory pathway *Nuclear Factor-kappa-light-chain-enhancer of activated B cells B (**NF-κB**)* by free-fatty acids in obesity will result in inhibition of AT macrophage ***autophagy***, a normal physiological process that maintains homeostasis, preventing the autophagic capacity of regulating inflammation, which in turn will promote the polarization of non-inflammatory macrophages to a M1 pro-inflammatory state. We propose that the "missing link" between these two pathways is the *mammalian target of rapamycin* (**mTOR**), which has been proposed as a negative regulator of autophagy, and in certain tumors, its activation is Inhibitor of Kappa B kinase beta (IKK)-dependent. We tested this hypothesis using a murine macrophage cell line, RAW264.7, and exposed these cells to a lipid-rich microenvironment to mimic the cellular metabolic inflammation observed in obese AT. Immunoblotting showed that treatment of macrophages with saturated fatty acids such as palmitic, myristic, and stearic acid, resulted in the upregulation of the functional marker of M1 pro-inflammatory phenotype IL-1β. This polarization was mediated by NF-κB and mTOR pathway as shown by the increase levels of phospho-NF-κB p65 and phospho-mTOR compared to untreated cells. This was accompanied by a decrease of autophagy markers Light Chain 3 II (LC3II) and Beclin. We also found that the interplay between NF-κB and Autophagy pathways is TSC1/mTOR-mediated as shown by the rescue of autophagy after NF-κB activation was blocked using BOT-64, an IKKβ inhibitor, and this was accompanied by the recovery of TSC1 activity and the decrease of phospho-mTOR in the presence of BOT-64.

P2310/B562

Compulsive Oxycodone Self-administration Potentiates Cell Signaling in the Rat Striatum Via the Mitogen-activated Protein Kinase (MAPK) Signaling Cascade.

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Objectives: Oxycodone is a prescribed synthetic opioid drug whose abuse has reached epidemic proportion in the USA because of over-prescription of the drug for pain related to cancer and other medical problems. Although oxycodone has been prescribed for a long time, much remains to be done to understand the basic neurobiological effects that render the drug so addictive to humans. We reasoned that oxycodone-induced adaptations in signaling pathways might be one of the mechanisms involved in its addictive properties. In the present study, we thus sought to identify changes in the

phosphorylation states of striatal proteins involved in the MAPK pathway of rats during early withdrawal from escalated and non-escalated oxycodone self-administration. **Methods:** Male Sprague-Dawley rats were trained to self-administer oxycodone or saline by pressing a lever to receive 0.1 mg/kg/injection via an intravenous catheter for 3-hours during the first week of self-administration training. At the end of the first week, the oxycodone animals were split into 2 groups: (1) short access (ShA) rats that continue to self-administer drug for 3 hours throughout the study (4 weeks); (2) long access takers (LgA) rats that self-administer drug for 6-9 hours for the last 3 weeks of the study. At the end of the study, rats were euthanized 2h after the last self-administration session. We then isolated striatal proteins to measure the expression and phosphorylation states of proteins involved in the (MAPK) signaling cascade including PKC, ERK1 and 2, MSK1 and 2, and CREB. **Results:** Rats given long access, but not short access, to oxycodone escalated their intake of oxycodone to different levels, with some rats (LgA-H) taking very large amounts of oxycodone and others (LgA-L) taking much less drug. Biochemical analyses revealed increased abundance of several phosphoproteins of the MAPK signaling cascade in only the LgA-H animals. There was increased PKC phosphorylation in the LgA-H Group. Increased MSK1 phosphorylation was observed in both LgA-L and LgA-H groups whereas only the LgA-H group showed increased MSK2 phosphorylation. Activation of the cascade also led to increased phosphorylation of CREB that is a target for the MSK proteins. **Conclusion:** These observations suggest that escalation of oxycodone intake that may lead to addiction in humans activates the MAPK signaling cascade in the rat brain. Identification of genes and proteins that are targets of this cascade should help to develop better therapeutic agents to combat oxycodone use disorder. Acknowledgement: This work is supported by DHHS/NIH/NIDA/IRP.

P2311/B563

Harnessing Single-cell an alysis to Discover Kinase Network Vulnerabilities in Colon Cancer.

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Colon cancer is the second deadliest cancer affecting both men and women. Despite its pervasiveness and active research, new therapies developed over the past two decades have failed to be effective. One reason for this failure is due to tumor cell heterogeneity. Tracking single cell responses to drug treatment is critical for identifying the next generation of therapies that are effective against colorectal cancer. We study cancer at the single cell and sub-cellular level using automated microscope and software customized in our lab. We focus on characterizing novel protein kinases that colon cancer cells use to grow and resist therapeutic intervention. Kinases transmit cellular signals that play a key role in various cellular processes such as cell division, metabolism, survival, and more. Their dysregulation has been found in all forms of cancer including those arising from the colon, brain, lung, breast, prostate, and other organs. Therefore, kinases are regarded as important effectors in human pathology and represent strong candidates for therapeutic targeting. Understanding the characteristics of protein kinases can pave the way for effective cancer diagnosis and treatment. Here, we 1) characterize the function of under-studied protein kinases, 2) determine the heterogeneity of the cells by loss of kinase function. We combine cancer biology, automated microscopy, high-throughput image processing, and computational methods to quantify hundreds of cell-level “features” such as size, shape and use this information for mapping the cellular response to drugs treatments. In order to study the large sets of protein kinase networks, we use RNAi assay to knock down one kinase at a time in normal colon cells, with total of 489 kinases. Images of cells are captured using high throughput microcopy. Then, we process and segment the images of cells using Cell Profiler software. Next, we quantify their shape and texture using TISMorph software[1] to capture > 500 different cellular “features” quantifying the

response of cells to the loss-of-function of individual protein kinases. The experiment is done in triplicates and we show that the data is reproducible. Using hierarchical clustering we group the kinases based on their similarity in their "features". Then we map kinases with unknown functions to pathways populated with well-characterized kinases based on their similarity in their features. Thus, identifying new kinase function. In addition, the kinases which their loss of function lead to heterogeneous morphometric response of the cells are identified. **References** [1] E. Alizadeh, W. Xu, J. Castle, J. Foss, and A. Prasad, "TISMorph: a tool to quantify texture, irregularity and spreading of single cells," *PLoS One*, vol. 14, no. 6, p. e0217346, Jun. 2019.

P2312/B564

Phosphatidylinositol 4-kinase Ii β Down Regulation Modulates Redox Signalling and Increases Radio-sensitivity in Mcf-7 Cells.

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Canonically type II phosphatidylinositol 4-kinases (PtdIns 4-kinase II) were involved in growth factor receptor signalling and vesicular trafficking. Recently these enzymes were shown to modulate apoptosis, cell adhesion, motility, and inflammatory responses in different cell types. Some of these effects were closely linked to redox homeostasis and ER stress. Involvement of PtdIns 4-kinase II β in these signalling pathways were investigated in human breast cancer cell line (MCF-7 cells) with the help of shRNA. Knockdown of PtdIns 4-kinase II β was correlated with increased ROS levels and decreased GSH GSSG ratio. Diphenyleneiodinium reduced ROS production suggesting a role for NADPH Oxidase. Concomitantly, these transfectants showed inhibition of NF-kappa B p65 translocation to nucleus and double-stranded DNA damage. In addition, these cells showed an increase in ER stress protein markers PERK, IRE-1α, BiP and PDI. Quantitative real time polymerase chain reaction experiments showed up regulation of GADD153 and down regulation of Bcl-2 and AKT3 mRNAs. At cellular level, these cells showed ER deformity. ROS scavengers, Caspase 9 and 12 inhibitors, ER-chemical chaperone rescued MCF-7 cells from PtdIns 4-kinase II β shRNA induced apoptosis. Gamma-irradiation of MCF-7 cells showed up regulation of PtdIns 4-kinase II β mRNA levels and PtdIns 4-kinase II β shRNA increased the radio-sensitivity. These results have potential implication in radiation therapy for cancer management.

P2313/B565

The Role of Selected Cell Signaling Pathways in Dibutyltin- Induced Synthesis of Interleukin-1 Beta.

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Dibutyltin (DBT) is an environmental contaminant primarily used to stabilize PVC plastic (polyvinyl chloride). It has also been used to deworm poultry. As a result of PVC plastics being used in pipelines and storage containers, DBT has been in drinking water, beverages, and seafood. The serum levels of DBT have been found in individuals as high as 0.3µM. Preliminary studies have shown that DBT induces increases of interleukin- 1 beta (IL-1β) production (intracellular + secretion) at lower concentrations and at higher concentrations it has shown decreases of IL-1β production in monocyte depleted peripheral blood mononuclear cells (MD- PBMC). IL-1β is a pro inflammatory cytokine that enables the human body to heal tissue if the body is injured or infected. Inappropriately elevated levels of IL-1β can cause chronic inflammation which is linked to cancer, autoimmune diseases, and diabetes. This study examines the role of certain cell signaling pathways that control immune cell production of IL-1β for

their role in DBT-induced increases in IL-1β production. The pathways that are examined are the mitogen activated protein kinases (MAPK) ERK 1/2 and p38 pathways. MD- PBMCs were exposed to the pathway inhibitors 1 hour prior to being treated with DBT at the various concentrations (0.25, 0.10, and 0.05). ERK 1/2 pathway was inhibited with the MEK inhibitor PD98059, and the p38 pathway was inhibited with the MEK inhibitor SB202190. The results of this study indicated that DBT utilized both the p38 pathway and ERK 1/2 pathway (cells from 5 separate donors) to stimulate increased IL-1β production. However, DBT appeared to rely more heavily on the p38 cell signaling pathway than the ERK 1/2 pathway to stimulate IL-1β produciton.

P2314/B566

Examining the Roles of Erk1/2 and P38 MAPK Pathways in the Dibutyltin-induced Increased Production of Interleukin-6 in Human Immune Cells.

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Dibutyltin (DBT) is a compound that is used to prevent parasitic diseases in certain poultry, and it is also used in the manufacturing of plastics. DBT has been found in drinking water and seafood due to leaching from polyvinyl chloride plastics used in water pipelines and storage containers. Exposure to DBT results from the ingestion of these contaminated foods and beverages and consequentially has been found in humantissues with concentrations as high as 0.3 μM. Previous studies indicate that in human immune cells, DBT can increase and decrease the secretion of the pro-inflammatory cytokines; tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), interleukin 1 beta (IL-1β), and Interleukin-6 (IL-6). Overproduction or abnormal levels of IL-6, can lead to chronic inflammation and inflammatory diseases such as Rheumatoid Arthritis, Lupus Erythematosus, and Crohn's Disease. To determine whether DBT is affecting the immune cell's ability to produce IL-6 or solely the secretion of this protein, in a previous study, the cellular production (both intracellular and secreted levels of IL-6) were investigated by using Enzyme Linked Immunosorbent Assays to measure secretion of IL-6 and Western Blots to measure intracellular levels of IL-6. The results indicate that when monocyte-depleted peripheral blood mononuclear cells (MD-PBMCs) were treated with DBT for a period of 24 hours with concentration ranging from 5-0.05 μM of DBT, there was an increased production of IL-6 in the lower concentrations (0.25-0.05 μM) in the cells from all donors. The ERK1/2 and p38 MAPK pathways regulate the production of IL-6, therefore the objective of this study was to examine their role in DBT-induced increases in IL-6 production by using the MEK inhibitor PD98059 to block the ERK 1/2 pathway and the SB202190 inhibitor to block p38 MAPK activity. When the MD-PBMCs were exposed to the inhibitors one hour prior to the 0.25-0.05 μM of DBT treatment, the results show that DBT-induced increases in IL-6 were dependent on both the p38 and ERK 1/2 MAPK pathways. DBT stimulates pathways that should normally be activated in response to infection or injury leading to inappropriate elevation of the potent inflammatory stimulus IL-6. Supported by NIH grant 2U54CA163066.

P2315/B567

Activation of Nrf2/HO-1 by a Novel Peptide Yd1 Attenuates Inflammatory Symptoms through Suppression of TLR4/MYyD88/NF-kB Signaling Cascade.

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We found that a multifunctional peptide YD1, purified from the strain *Bacillus amyloliquefaciens* from kimchi, possessed potent anti-inflammatory activity. The modulatory effect of YD1 on the toll-like receptor 4 (TLR-4) / Myeloid differentiation primary response 88 (MyD88) / nuclear factor kappa B (NF-κB) and Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway, was investigated in lipopolysaccharide (LPS) stimulated RAW264.7 cells and carrageenan (CA)-induced mice model. YD1 suppressed the magnitude of nitric oxide (NO) and prostaglandin E2 (PGE_2) by down-regulating inducible nitric oxide synthase (iNOS) and cyclooxygenease-2 (COX-2). YD1 also down-regulated the mRNA levels of macrophage biomarkers (e.g., IL-1β, IL-6, and TNF-α,) and inhibited nuclear translocation of NF-κB in the RAW264.7 cells as well as in CA-induced inflammatory mice. Our results concluded that YD1 might control macrophage polarization through modulating the cross-talk between the following three mechanisms: 1) attenuating LPS-induced nuclear translocation of NF-κB; 2) activation of Nrf2 and consequently inducing HO-1 expression. 3) HO-1 selective inhibitor SnPP diminished the inhibitory effects of YD1 on the activation of the NF-κB pathway and the pro-inflammatory cytokines. Collectively, YD1 exhibited anti-inflammatory activity via suppressing pro-inflammatory macrophage polarization. Therefore, our findings provide new evidence for the potential of YD1 in the treatment of chronic inflammation related diseases and oxidative stress.

P2316/B568

Inhibition of Melanogenesis by *Syzygium Cumini*Seed Extract Via Regulation of MAPKs/MITF Signaling Pathway.

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Melanin, the primary factor of skin color, is chiefly produced in response to ultraviolet (UV) irradiation and make defense against the detrimental effects of UV into skin, but its overproduction causes hyperpigmentation disorders such as freckle, hyperpigmentation, and melasma. In the current study, several whitening materials have cytotoxicity to the skin, therefore the development of natural ingredients without reverse effects is necessary. Hence, the aim of this study was to investigate the efficacy of the ethanolic extracts of *Syzygium cumini* seeds (CSEE) against melanogenesis and sought to its underlying mechanisms in Melan-a cells. CSEE significantly inhibited the mushroom tyrosinase activities in a dose-dependent manner. CSEE treatment also meaningfully suppressed the melanin content as well as the cellular tyrosinase activity in Melan-a cells. Furthermore, CSEE treatment strongly abolished the expression of microphthalmia-associated transcription factor (MITF) and its downregulated protein such as tyrosinase (TYR) and tyrosinase-related protein-1 (TYRP-1). CSEE treatment caused the activation of MAP kinase phosphorylation (ERK1/2 and p38), leading to down-regulation of tyrosinase and MITF, resulting in the attenuation of melanin production in Melan-a cells. An d then, treatment of U0126 (ERK1/2 inhibitor) and SB202190 (p38 inhibitor) restored the expression

of protein that was decreased treated with CSEE. In addition, through HPLC analysis and single compound tyrosinase inhibitory assay analysis, we knew quercetin, chlorogenic acid, coumaric acid, and caffeic acid were contributed to inhibitory activity of CSEE. Collectively, these results suggested that CSEE could be a potential natural source for the development of new anti-melanogenic agents.

P2317/B569

Protective Effects of a Chinese Black Tea Aqueous Extract on UVB-irradiated Photoaging Via MAPKs/Nrf2-mediated Down-regulation of MMP-1 in Keratinocyte Cells.

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Skin photoaging is mainly caused by ultraviolet B (UVB) irradiation, related with stimulation of collagen degradation and up-regulation of matrix metalloproteinases (MMPs). Fuzhuan-brick tea (FBT) is used as a beverage and as a nutritional supplement by the ethnic groups of Southern/Western China. The aim of this study was to investigate the anti-photoaging effects of Fuzhuan-brick tea aqueous extract (FBTA) in UVB-mediated HaCaT cells. FBTA exhibited potent antioxidant activity and ameliorated oxidative stress by suppressing intracellular reactive oxygen species (ROS) generation without any toxicity. FBTA activated messenger RNA (mRNA) and protein expression levels of antioxidant enzymes and phase II detoxifying enzymes, specifically heme oxygenase-1 (HO-1). FBTA up-regulated the nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated pathway in HaCaT cells through the phosphorylation of p38 and extracellular signal-regulated kinase (ERK). FBTA also down-regulated the expression of MMP-1, whereas up-regulating type I procollagen production via Nrf2 signaling pathway. Overall, these results suggested that FBTA has potential to be a great functional ingredient for the preparation of UVB-stressed skin photoaging, in that the remedy can be applied for the development of cosmetic products and medicines.

P2318/B570

***Spatholobus Suberectus* Stem Extracts: Inhibition of UVB-induced Skin Aging by Modulating MAPK/AP-1 Signaling-mediated Down-regulation of MMPs.**

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Traditionally, the stem of *Spatholobus suberectus* has been applied to treat inflammation-induced thrombosis. It has also been reported that *Spatholobus suberectus* have the potential to regulate cartilage-related MMPs and TIMPs and anti-inflammatory activity. In this presentation, we demonstrated that aqueous and ethanolic extracts of the stem of *Spatholobus suberectus* (SSW and SSE) inhibited UV-induced skin photo-aging in HaCaT cells. SSW and SSE extracts were identified by HPLC and they have the major active components which were gallic acid, catechin, vanillic acid, syringic acid and epicatechin. SSW and SSE showed that significantly decreased the elastase enzyme activity and quenched UVB-induced generation of cellular reactive oxygen species in the cells. Our results confirmed that SSE could regulate the expression of MMPs, TIMP-1, COL1A1, ELN and HAS2 at their transcriptional and translational level and suppress the UVB-induced phosphorylation of MAP kinases, c-Jun and NF-κB.

Furthermore, we found that a combination of syringic acid, epicatechin and vanillic acid revealed strong synergistic effects on elastase inhibitory activity in which the combination index was CI= 0.28. Collectively, our results indicate that SSE might be useful as a natural ingredient for the remedy of UVB-induced skin aging.

P2319/B571

Effects of Crowding Agents and Osmolytes on Wnk1 and Wnk3 Kinases.

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WNK kinases are major regulators of electro-neutral cotransporters that control blood pressure and cell volume. The regulation of these cotransporters occurs via the phosphorylation by WNKs and their substrate OSR1 (Oxidative Stress Responsive Kinase 1). Mutations in *WNK1 and WNK3* genes were determined to cause Familiar Hyperkalemic Hypertension (also known as pseudohypoaldoseronism II, or Gordon's Syndrome) and breast cancer, respectively. A goal of this study is to explain how osmotic stressors affect WNKs activity. Our previous studies determined that WNK kinases are inhibited by chloride and activated by osmotic stress induced by crowding agents (such as PEG400, Ficoll, hemoglobin) or osmolytes (such as sorbitol, sucrose). Based on DSF (Differential Scanning Fluorimetry), we saw stability differences in WNK kinases caused by these small molecules. We also demonstrated that crowding agents and osmolytes induce auto-phosphorylation of WNKs 1 and 3. These actions were opposed by chloride. Understanding the molecular mechanism of WNK kinases osmotic sensing will be helpful in finding potential drug targets for hypertension, breast cancer and related disorders.

P2320/B572

Do Strad Pseudokinases Specify the Lkb1 Functions? the Ampk Activation Case.

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Currently, there is no doubt that the LKB1 kinase, causally linked to the Peutz-Jeghers syndrome, is at the center of an important signalling node affecting numerous cellular processes whose deregulation contributes to diseases such as cancer. Although downstream signalling events of this kinase were extensively studied, signals that regulate LKB1 functions remain almost completely uncharacterized. This question has a fundamental relevance and has to be addressed to improve our understanding of LKB1-associated disorders. Structurally, LKB1 is composed of a serine-threonine kinase domain with poor catalytic activity flanked by a C-terminal CAAX sequence that serves as a prenylation site, and a putative N-terminal nuclear localization sequence (NLS). Full LKB1 kinase activity requires its interaction with STRAD (STE-20 Related ADaptator) proteins (STRADα or STRADβ), which are ubiquitously expressed pseudokinases. Since STRAD pseudokinases reveal the LKB1 kinase activity, they appear to be the first level of LKB1 complex regulation. Hence, we have decided to address whether STRAD pseudokinases specify LKB1 functions. After defined the relative abundance and LKB1 activity related to each STRAD paralogues we assessed involvement of each STRAD in phosphorylation and activation of the best characterized LKB1 substrate: AMPK. Our results establish LKB1/STRADα complex is the most abundant and has a preponderant role in LKB1 stabilization and AMPK activation. Indeed, in several cell types invalidated for each STRAD, individually or concomitantly, derived from Knock out mouse models or cell lines genetically modified by CripRCas9, we demonstrate that LKB1/STRADβ has fewer catalytic activity toward AMPK, in *vitro* and in *vivo,* than the LKB1 complex formed with STRADα. To conclude, our

results, demonstrate that both LKB1 complexes comprised either STRADα or its paralogue STRADβ have specific features such as catalytic activity efficiency towards substrates, role in LKB1 stability and beyond in cell signalling consequences. Although some functions or substrates should be shared by both complexes, STRAD pseudokinases appear to determine specific LKB1 functions.

P2321/B573

Examining the Role of MTORC2 at Mitochondria.

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Mammalian mechanistic target of rapamycin (mTOR) kinase coordinates cellular anabolic reactions with the availability of cellular nutrients. In cells, mTOR exists in the context of two structurally and functionally distinct complexes, mTORC1 and mTORC2. While mTORC1 is the subject of intense research, the regulation and cellular functions of mTORC2 are not well understood. We have previously characterized the intracellular localization and regulation of the endogenous mTORC2 by PI3K (Ebner *et al*., JCB 2017). Among other cellular membrane compartments, we and others have reported mTORC2 activity at the mitochondria, although its exact function and targeting to mitochondrial membranes remain enigmatic. Here, we examined mitochondrial respiration in mouse embryonic fibroblasts obtained from the wild type and mSin1-knockout mice (a gift of Dr. Estela Jacinto, Rutgers). We show that mSin1$^{-/-}$ MEFs demonstrate significantly higher basal oxygen consumption rate (OCR) and spare respiratory capacity than the wild type cells, though both WT and mSin1$^{-/-}$ MEFs have similar mitochondrial mass. The observed effects were Akt- and rapamycin-independent, but sensitive to mTOR inhibitor Torin1, suggesting the effects were mTORC2-specific. Further, in addition to the obligate components of mTORC2 - mTOR, mRictor and mLST8 - LC-MS/MS analysis of the mSin1-GFP interaction partners identified a number of mitochondrial membrane transporters, including SLC25A3, SLC25A5 and SLC25A6, involved in the control of oxidative phosphorylation. Inhibition of the ADP/ATP translocase by carboxyatractyloside increased mTORC2 activity at mitochondria in HEK293T cells and attenuated basal respiration in WT MEFs. Our results suggest that mTORC2 controls the efficiency of mitochondrial respiration. We propose that in addition to control of the mitochondrial hexokinase, mTORC2 could modulate the efficiency of oxidative phosphorylation through the control of mitochondrial transporters, including those of the SLC25 family.

P2322/B574

Insp8 Regulates Cellular Phosphate Homeostasis and Calcification.

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Phosphate (Pi) has multiple functions that direct the survival of all living organisms. It is required for mineralization of cartilage, bone, and dentin. In its organic form, Pi is a component of genomic material, it serves as an energy currency, and it is ubiquitous in cell signaling. Thus, regulation of Pi uptake and efflux pathways across cells is essential to life, but the underlying mechanisms in humans are largely unknown. Here, we demonstrate that Pi is regulated by 'IP8', an inositol pyrophosphate (PP-IP) cellular signal. We found Pi efflux was blocked when cellular [InsP8] was reduced, either genetically (KO of the diphosphoinositol pentakisphosphate kinases (PPIP5Ks) that synthesize InsP8), or pharmacologically (cell treatment with 2.5 µM of dietary flavonoid, or 10 µM N2-(m-trifluorobenzyl), N6-(p-nitrobenzyl) purine, to inhibit inositol hexakisphosphate kinases upstream of PPIP5Ks). Significantly, attenuated Pi efflux by PPIP5K KO cells was quantitatively phenocopied by KO of XPR1 itself. Moreover, Pi efflux from PPIP5K

KO cells was rescued either by stable transfection of wild-type PPIP5K1 (a kinase-dead mutant was inactive), or, in a dose-dependent manner, by liposomal delivery of a metabolically-stable, methylene bisphosphonate (PCP) analogue of InsP8; PCP-analogues of other PP-InsPs were all ineffective. To place these observations in a cellular biology context, we studied differentiation of the Soas-2 osteosarcoma cell-line. Loss of XPR1-mediated Pi efflux in PPIP5K KO cells enhanced a differentiation program: elevated expression of osteocalcin, osteonectin and alkaline phosphatase, and acceleration to the mineralization endpoint. Our data suggest catalytically-compromising PPIP5K mutations might extend the genetic repertoire for XPR1 dysregulation; PPIP5Ks may be therapeutic targets for bone maintenance.

P2323/B575

Direct Mitochondrial Transfer Improves Insulin Resistance through Ameliorating Mitochondrial Dysfunction in C2c12 Myotubes.

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Mitochondrial dysfunction in muscle is associated with insulin resistance in patients with type 2 diabetes mellitus (T2DM). Several studies attempted that insulin resistance was reduced by improving mitochondrial dysfunction. However, there are few studies whether direct mitochondrial transfer improve insulin resistance in muscle cell. The aim of this study was to evaluate the efficacy of direct mitochondrial transfer for insulin resistance in C2C12 myotubes. We prepared a model of insulin resistance by treating palmitic acid (PA) in mouse skeletal muscle cell line (C2C12). Then, mitochondria-derived from C2C12 cells were centrifuged with host cells to investigate changes in IR. We compared changes in glucose uptake and insulin signal pathway-related proteins. The induction of IR was verified by glucose uptake, and then the generation of oxidative stress by PA treatment was observed. As a result, oxidative stress and mitochondrial fragmentation are reduced through healthy mitochondrial transfer. Furthermore, this study reveals the improvement of insulin sensitivity by upregulating Glut4 expression, IRS1-Akt phosphorylation and mitochondrial dynamics-related proteins. Our results indicated that direct mitochondrial transplantation could attenuate insulin resistance. Therefore, understanding mitochondrial dysfunction responsible for IR is beginning to define the target for the treatment of T2D.

Signaling Receptors (RTKs and GPCRs)

P2324/B576

Regulation of Discoidin Domain Receptor 1 Kinase Activity by the Intracellular Juxtamembrane Region.

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Introduction the discoidin domain receptor (DDR) DDR1 is a collagen-binding receptor tyrosine kinase (RTK) which controls cellular proliferation and migration. Aberrant DDR1 signalling is implicated in the progression and poor prognosis of several diseases, including many cancers. DDR1 is therefore an attractive target for pharmacological intervention. However, unlike many other RTKs, the processes governing DDR signalling are poorly characterised. Our study aimed to better understand these processes by investigating the functional role played by the intracellular juxtamembrane (JM) region of

DDR1. **Materials and Methods** X-ray crystallography was performed on the intracellular domain of DDR1 with diffraction data collected at Diamond Light source. Immunoblotting of Baculovirus expressed DDR1 constructs was used to monitor receptor autophosphorylation over time, and the identity of phosphorylated tyrosine residues was confirmed by tandem mass-spectroscopy (MS/MS). Enzyme kinetics of each DDR1 phosphoform were determined using a luciferin/luciferase-based ADP-Glo™ assay. **Results** We solved a 2.5 Å resolution crystal structure of the DDR1 cytoplasmic domains. The structure revealed that the DDR1 JM region inserts into the kinase active site, disrupting the transition between inactive and active catalytic states. This JM autoinhibition is shown to be relieved through a concerted two-step process beginning with the phosphorylation of tyrosine residues within the JM region. This initial catalytic event enables kinase activation loop autophosphorylation and facilitates the transition to an active conformation. Through isolating different phosphoforms of DDR1 we also show that JM phosphorylation alone is sufficient to drive a marked increase in substrate affinity (Km decreases 100 fold). Subsequent activation loop phosphorylation results in a five-fold increase in catalytic rate. **Discussion** These data identify the DDR1 JM region as a novel regulator of signalling, highlighting that phosphorylation of this region is the critical first step in DDR1 activation. This study will aid future structure-guided design of inhibitors that can specifically target DDR1 and which may have therapeutic applications.

P2325/B577

Smoothened Stimulation by Membrane Sterols Drives Hedgehog Pathway Activity.

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Hedgehog (Hh) signaling within the primary cilium is fundamental to embryonic development and tissue regeneration. Aberrant signaling postnatally leads to several malignancies including basal cell carcinoma, the most common human cancer, and medulloblastoma, the most common pediatric brain tumor. At the ciliary membrane, Hh proteins bind to and inhibit the transmembrane cholesterol transporter Patched1 (PTCH1), permitting activation of the seven transmembrane (7TM) transducer Smoothened (SMO) via a poorly understood mechanism. Here, we present the crystal structure of active SMO bound to the agonist SAG21k and an intracellular-binding nanobody that stabilizes a physiologically relevant active state. An alogous to other G protein-coupled receptors (GPCRs), activation of SMO is associated with subtle motions in the extracellular domain and larger changes intracellularly. In contrast to recent models that have emphasized lipid binding to the SMO extracellular cysteine-rich domain (CRD), we find a cholesterol molecule critical for SMO activation bound deep within the 7TM pocket. Mutational analysis reveals that this 7TM sterol binding site is essential for SMO activation in cultured cell and in *vitro* systems, and is supported by molecular dynamics simulations of SMO-sterol interactions. We propose that PTCH1 inactivation enables a transmembrane sterol to access this 7TM site, potentially through a hydrophobic tunnel, driving SMO activation. Our present studies are now focused on three outstanding challenges: 1) biochemically identifying the endogenous molecule that binds the SMO 7TM sterol site under native physiological conditions; 2) understanding how SMO integrates the information relayed through its CRD and 7TM sterol binding sites to ensure appropriate levels of Hh pathway activity in vivo; 3) understanding how PTCH1 can selectively and specifically inhibit SMO without affecting other sterol-dependent signaling processes. These results delineate the structural basis for PTCH1-SMO

regulation, highlight the deep evolutionary connections between SMO activation and sterol biology, and suggest a new strategy to overcome clinical resistance to SMO inhibitors.

P2326/B578

Investigating the Mechanism of Modulating Hippo-yap Activities by Perturbing the Cellular Membrane Cholesterol Homeostasis.

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Objectives; Activation of hepatic stellate cell (HSC) is a major hallmark of liver fibrosis and requires Hippo-YAP signaling pathway activation. Notwithstanding that external stimuli are translated into the pathway through the plasma membrane where most of cellular cholesterol locates, how membrane cholesterol homeostasis modulates the process hasn't been extensively studied. **Methods;** Multiple reported stimuli were used as means to activate Hippo-YAP pathway. The activation of the pathway was addressed by target gene expression with RT-PCR and Hippo kinases activation with western blot. Several pharmacological reagents were used to modulate membrane cholesterol level and to modify cholesterol status. Furthermore, siRNA-, shRNA-mediated knock-down, ectopic expression, and chemical inhibitors were adopted to manipulate the functions and the expression of proteins. Various cell lines with different tissue origins were also used to confirm our findings. **Results;** Based on the previous reports, several stimuli were selected and tested to efficiently induce YAP activation in HSC; agonists of several GPCR family members, TGF-b1, and high external stiffness. By using siRNAs and antagonists, we also demonstrated that Spingosine-1-phosphate (S1P) induces YAP target gene expression through S1PR2 and Gα13 and Gαq/11 act at the downstream. Treatment with diverse cholesterol modulating agents, such as MβC, HPβC, and Fillipin, surprisingly synergistically enhanced YAP target gene expression upon several stimuli, which was efficiently reverted by supplementation of exogenous cholesterol. Western blot showed that dephosphorylation of Hippo pathway kinases induced by S1P were even more increased by MβC pre-treatment, indicating that perturbing the plasma membrane cholesterol level affect the canonical Hippo pathway. Furthermore, the MβC pre-treatment also showed the similar enhanced effect upon the stimuli that were known to directly induce Rho/ROCK/Hippo-YAP activation independent of membrane receptors, suggesting that the effect of MβC were not confined to the membrane level and were transmitted to the cytosol. Western blot and RT-PCR evidenced that two isoforms of ROCK were differently regulated by the cholesterol perturbing agents, at both transcription and post-transcription levels. **Conclusion;** Acute perturbation of membrane cholesterol homeostasis shapes Hippo-YAP signaling pathway through differently regulating ROCK-1 and ROCK-2.

P2327/B579

Rapid Regulation of Oxidative Phosphorylation by Insulin in Human Embryonic Stem Cells.

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Insulin is an extrinsic factor essential for human embryonic stem cell (hESC) maintenance and differentiation, and the functions are always carried out through transcription and translation over hours or even days. Emerging studies suggest that insulin is important for energy metabolism, which is increasingly recognized as an active control of cell functions, but in hESCs how insulin signal transduction influences energy balance and whether there is immediate impact are largely unknown. Here we show that the presence of insulin rapidly stimulates substrate-specific oxidative phosphorylation (OXPHOS) in

hESCs. Pharmacologic and genetic manipulations reveal that insulin regulates OXPHOS through PI3K/AKT/GSK3 cascade within minutes before significant changes in the expression of metabolic enzymes occur. Intriguingly, we observed that insulin-dependent OXPHOS is hESC-specific, and it diminishes in differentiated cell types. Moreover, we noticed that insulin or GSK3 inhibition could significantly suppress the activation of caspase. Collectively, these results suggest that insulin regulated OXPHOS in a rapid mode and insulin is continuously required to maintain full mitochondrial capacity in hESCs. Our findings provide molecular insights into the association of insulin signal transduction and mitochondrial energy metabolism, and highlight the necessity of insulin in the consistency and accuracy of metabolic analyses in intact hESCs.

P2328/B580

Nuclear HCAR1 Promotes Cancer Cell Migration.

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G-protein Coupled Receptors (GPCRs) are major class of receptors virtually involved in every biological processes. More than 40% of all pharmaceuticals target GPCRs indicating their pathophysiological significance. One of the most significant dimensions in GPCR biology is nuclear localization of certain GPCRs having different function than their surface counterpart. HCAR1 is a GPCR and the endogenous receptor of lactate. Lactate through HCAR1 can inhibit lipolysis and inflammation, promote angiogenesis and cancer progression. We hypothesized that HCAR1 has nuclear localization exerting different function in the nucleus. We performed immunofluorescent staining followed by confocal imaging, biochemical fractionation and electron microscopy to determine HCAR1 localization pattern. To investigate the potential translocation of HCAR1 from plasma membrane to the nucleus upon ligand binding, fluorescent activating peptide technology was adopted using a cell impermeable fluorogen pulse chase. Site directed mutagenesis identified HCAR1 domains involved in its differential localization. We screened for activation of potential intranuclear signaling pathways upon lactate stimulation on isolated nuclei and performed ChIP-seq with HCAR1 to elucidate its potential direct gene regulatory function. Our data shows intact HCAR1 is localized to the nucleus and nuclear membrane independent of ligand binding and ICL3 and phosphorylation of S305 in the C-terminal domain are responsible for this localization pattern. We show nuclear Akt, Erk1/2 and p38 phosphorylation upon lactate stimulation, and this phosphorylation pattern is lost in the nuclei of KD cells, proving nuclear HCAR1 activates intranuclear signaling pathways. ChIP data shows lactate induces redistribution of HCAR1 in the genome toward gene loci and transcription start sites. Ontological analysis indicates HCAR1 is enriched for genes involved in cellular movement upon lactate binding. Cell scratch assay proves KD cells have ~50% lower migration rate. While re-introduction of RNAi resistant WT HCAR1 rescued the migratory phenotype, rescue with Delta ICL3 or S305A mutants behaved like total KD demonstrating nuclear HCAR1 governs cell migration. Taken together, we prove nuclear localization of HCAR1 and provide evidence for its function as signaling receptor in the nucleus/nuclear membrane and direct gene regulatory functions. Previous studies have shown that HCAR1 has a crucial role in progression and metastasis of many cancers. Based on our data, we hypothesis that dual function of nuclear HCAR1 significantly contributes to its role in cancer pathology. Our next aim is to validate this hypothesis in animal model and determine nuclear HCAR1 contribution to these phenomena.

P2329/B581

Endosomal G Protein-coupled Receptor Signaling Modulates Liver Cell Metabolism.

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Upon activation by specific ligands, G protein-coupled receptors (GPCRs) signal via cyclic AMP (cAMP) production. The classical model of GPCR signaling describes that cAMP production occurs at the plasma membrane. However, work from our lab and others has revealed a new model of GPCR signaling, which includes cAMP production from the endosomal membrane. Using the Beta2-Adrenergic Receptor (B2AR) as a model GPCR, our lab has shown that endocytosis is a required component to achieve the cAMP production necessary for downstream transcription. Microarray analysis identified a subset of transcriptional targets whose level of expression were significantly endocytosis-dependent. The top transcriptional target identified was Phosphoenolpyruvate Carboxykinase 1 (PCK1), a major control point of gluconeogenesis. As these studies were completed in HEK293 cells, we were interested to explore these mechanisms in a more physiologically relevant system. We chose to pursue this line of experiments in liver cells, as it is known that GPCR signaling is intimately connected to metabolic regulation in this organ. We have chosen to study B2AR as well as the Glucagon Receptor (GCGR), both of which are Gscoupled GPCRs which signal through cAMP. qRT-PCR analysis of samples in the presence or absence of the drug Dyngo, a dynamin inhibitor, indicates that endocytosis-dependent transcription exists in primary mouse hepatocytes downstream of both B2AR and GCGR activation. Importantly, this transcriptional mechanism includes regulation of key gluconeogenic genes PCK1, G6PC, and PGC1α. Current work is focused on examining how B2AR and GCGR endosomal signaling and trafficking impact metabolic regulation. Future work will examine how endosomal GPCR signaling is altered in hepatocytes from animals with dysregulated metabolism, such diabetes or non-alcoholic fatty liver disease.

P2330/B582

Fgf Signaling Promotes Myoblast Proliferation through Activation of Wingless Signaling.

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Indirect flight muscles (IFMs) are the largest muscles in *Drosophila* and are made up of about 3000 myonuclei per fiber. The generation of these giant muscles requires a large pool of adult muscle precursors (AMPs). The myoblasts that give rise to these muscles are associated with larval wing imaginal disc, and increase in number from 10-12 in first instar larvae to over 2500 by the end of the third larval instar. Previous studies have shown that Wingless (Wg) and Notch signaling are required for the regulation of myoblast proliferation. However, other factors that control the scaling up of the myoblast pool are largely unknown. Here, we examine the role of Fibroblast growth factor (FGF) signaling in the proliferation of wing disc associated myoblasts. First, we visualized the accumulation of different components of the FGF signaling cascade in the wing imaginal disc We find that the FGF receptor (Heartless) and downstream effectors (Dof and Pointed) are present in myoblasts, whereas, FGF ligands (Thisbe and Pyramus) are expressed in surrounding epithelial cells of the wing disc. Next, we show that attenuation of FGF signaling results in diminished myoblast pool. This reduction in the pool size is due to decreased myoblast proliferation. By contrast, activating FGF signaling increases the myoblast pool size and restores the proliferative capacity of *htl* knockdown flies. To further dissect the function of FGF signaling in the amplification of the AMPs, we examined cross-talk between FGF and Wg signaling. Our results demonstrate that Heartless acts through up-regulating β-catenin/armadillo to promote myoblast proliferation. Finally, we show that FGF mediated regulation of myoblast

proliferation is dependent on Cyclin E expression. Our studies identify a novel role for FGF signaling during IFM formation and uncover the mechanism through which FGF coordinates with Wingless signaling to promote myoblast proliferation.

P2331/B583

Fgfr Activates Non-canonical Tgfβ Signaling in Kinase-independent Manners, Contributing to Thyroid Eye Disease.

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FGFR activates non-canonical TGFβ signaling in kinase-independent manners, contributing to thyroid eye disease. Chien-Hsiang Chou[1], Ting-Xuan Lu[1], Er-Yen Yen[7], Shyang-Rong Shih[2, 3,4], Chih-Wei Shih[6], Yi-Hsuan Wei[5], Shu-Lang Liao[5] and Feng-Chiao Tsai[1,3] [1]Institute of Pharmacology, National Taiwan University College of Medicine, Taipei, Taiwan. [2]Department of Internal Medicine, National Taiwan University College of Medicine, Taipei, Taiwan. [3]Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan. [4]Center of anti-Aging and Health Consultation, National Taiwan University Hospital, Taipei, Taiwan. [5]Department of Ophthalmology, National Taiwan University Hospital, Taipei, Taiwan. [6]Department of Ophthalmology, Zhongxing Branch, Taipei City Hospital, Taipei, Taiwan. [7]Department of Genomic Medicine, University of Texas MD an derson Cancer Center, Houston, TX, USA. Thyroid eye disease (TED), or Graves' ophthalmopathy, is an autoimmune disorder caused by excessive extraorbital fibrosis and adipogenesis, both of which are regulated by orbital fibroblasts. Recent therapeutic advances indicated the potential of targeting growth factors including IGFs and VEGFs to treat TED. We thus explored if the major growth factor of fibroblast-FGF-was also involved in TED. Our recent results revealed high serum FGF1 and FGF2 levels in TED patients. We also noticed that FGF increased lipogenesis and decreased fibrogenesis in HS68 cells and orbital fibroblasts, indicating the importance of FGFR signaling in the pathogenesis of TED. We further elucidated how FGFR signaling altered the differentiation of orbital fibroblast. Both literature results and our preliminary findings revealed that the kinase activity of FGFR suppressed TGFβR signaling, reducing α-SMA formation and fibrogenesis. Interestingly, unlike FGFR inhibitors that increased α-SMA levels in fibroblasts, FGFR depletion via knockdown abolished the effect of TGFβ to increase α-SMA, indicating that FGFR might also alter TGFβ signaling in kinase-independent manners. Moreover, the canonical TGFβ pathway via Smad3 activation was unaffected during FGFR knockdown, suggesting the kinase-independent role of FGFR on TGFβR was through non-canonical TGFβR signaling. We are currently working on the mechanisms of this kinase-independent non-canonical FGFR-TGFβR signaling, with the hope to improve current treatment for TED in the near future.

P2332/B584

Function Study of Leg1 on Protection of Liver Development of Zebrafish Under Oxidative Stress.

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Function study of Leg1 on protection of liver development of zebrafish under oxidative stress Bai Yun [1], Hu Minjie [1], Wang Jinyang [1], Huang Heping [1], Xie Aixuan [1], Peng Jinrong [1] *and Chen Jun [2] * 1 College of an imal Sciences, 2 College of Life Sciences, Zhejiang University, Hangzhou, China pengjr@zju.edu.; chenjun2009@zju.edu.cn Unlike mammals and birds, teleost fish undergo external embryogenesis, and therefore their embryos are constantly challenged by stresses from their living environment. These stresses, when becoming too harsh, will cause arrest of cell proliferation, abnormal cell death or senescence. Such organisms have to evolve a sophisticated anti-stress mechanism to protect the process of embryogenesis/organogenesis. However, very few signaling molecule(s) mediating such activity have been identified. liver-enriched gene 1 (leg1) is an uncharacterized gene that encodes a novel secretory protein. In the zebrafish genome, there are two copies of leg1, namely leg1a and leg1b. In this report, we show that leg1a mutant exhibits a stress-dependent small liver phenotype that can be prevented by chemicals blocking the production of reactive oxygen species. Further studies reveal that Leg1a binds to FGFR3 and mediates a novel anti-stress pathway to protect liver development through enhancing Erk activity. This finding may explain the adaption of teleost fish in coping with environmental changes.

P2333/B585

Genome-Wide CRISPR/Cas9 Screening Towards Identification of the Lacritin Homeostasis Receptor.

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Introduction: Homeostasis of the surface of the eye appears to be controlled almost entirely by lacritin, a prosecretory mitogenic glycoprotein constituent of tears. In mouse dry eye and a human dry eye-like cell system, exogenous lacritin restores homeostasis by transiently accelerating epithelial autophagy and restoring oxidative phosphorylation through mitochondrial fusion. Although we understand in general how lacritin works, many details are missing - including the signaling receptor. Lacritin pro-homeostatic and mitogenic signaling in corneal epithelia involves Ca^{2+} signaling through calcineurin, AKT, FOXO1/ATG7 and FOXO3/ATG101. Rather than acting alone, the unknown receptor partners with a transiently deglycanated form of the cell surface proteoglycan syndecan-1. To capture these details that could be relevant to drug cross-reactivity, our goal was to identify the lacritin main receptor using a genome-wide CRISPR/Cas9 screen of human corneal epithelial cells (HCET). **Methods:** HCET cells were transduced at MOI of 0.3 with lentiviral Human CRISPR Knockout Pooled Library that comprises four sgRNAs targeting each of 19,114 annotated human protein coding genes. We then model the loss of homeostasis in dry eye by introduction of IFNG and TNF, followed by rescue with 'N-94', a synthetic peptide representing lacritin's C-terminal active domain. sgRNA's in cells that could no longer respond to N-94 were subjected to PCR amplification and next generation sequencing. The change in abundance of perturbations from sample to sample was then calculated using log-normalized data to generate log-fold-change (LFC) values, displayed in volcano-plots. **Results:** Our screen revealed multiple genes involved in lacritin pro-homeostatic signaling. One first screen hit was the G protein coupled receptor 87 (GPR87). Validation studies demonstrated that knocking down GPR87 expression prevents rescue of HCET cells stressed with IFNG and TNF by N-94. These data indicate that GPR87 has a critical role in lacritin homeostasis signaling. Our screen also identified many key players in the Hippo Pathway

including LATS1 and the transcription factor TEAD4. ShRNA knockdown of TEAD4 expression also abrogated lacritin homeostasis activity, suggesting a role for the Hippo Pathway in the lacritin homeostasis regulation. Further validation assays using GPR87$^{-/-}$ and TEAD4 $^{-/-}$ cells are under way.

P2334/B586

Investigating the Mechanism of Cytoplasmic Receptor Tyrosine Kinase Signaling.

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Receptor tyrosine kinases (RTKs) are a family of transmembrane receptors that activate signaling cascades to control cell proliferation, differentiation and growth. The classical model states that upon ligand binding, RTKs become dimerized leading to autophosphorylation and recruitment of downstream effectors to activate various signaling pathways, including the Ras/Erk mitogen-activated protein kinase (MAPK) pathway. In some cancers, chromosomal translocations lead to changes in RTK structure and localization that turn a plasma-membrane bound receptor into a cytosolic-localized kinase fused to a higher-order oligomer clustering partner. While these translocations are well-documented, how cytosolic receptors are able to activate downstream signaling remains unclear. To further investigate this question, we have created cytosolic versions of these receptors that are fused to an optogenetic construct that oligomerizes in response to blue light. Through our system, we found that some receptors are able to signal from the cytosol, such as fibroblast growth factor receptor (FGFR), and others are only able to signal when bound to the plasma membrane, such as epidermal growth factor receptor (EGFR). Interestingly, we find that although EGFR does not trigger downstream signaling, it autophosphorylates in response to oligomerization which is generally thought to be the first step in signal pathway activation. We predict that these differences in signaling arise from contact with downstream adaptor proteins, such as FGFR's interaction with membrane-bound fibroblast growth factor receptor substrate 2 (FRS2). Subsequent experiments and analysis with our system will establish how various RTKs are able to induce and maintain MAPK signaling.

P2335/B587

An Unexpected Role for Ripk3 in Regulating Growth Factor Receptor Expression and Signaling in Endothelial Cells.

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Receptor-interacting protein kinase 3 (RIPK3) is considered to be an essential mediator of necroptosis, which is a form of programmed necrotic cell death. However, other roles for RIPK3 have started to emerge in recent years, including roles in inflammatory cytokine production and cellular metabolism. Curiously, RIPK3 is highly and specifically expressed in endothelial cells (ECs) during murine embryonic vascular development, but our lab recently reported that excessive RIPK3 is detrimental to murine vascular integrity at midgestation in a necroptosis-independent manner. However, the mechanism by which RIPK3 regulates vascular integrity is unclear. We first questioned whether RIPK3 might impact vascular sprouting, which is active at the embryonic time point at which RIPK3 protein is highly expressed in ECs in vivo. Indeed, we found that RIPK3 knockdown attenuated vascular sprouting in an in vitro sprouting assay using human umbilical vein endothelial cells (HUVECs). Since vascular endothelial growth factor a (VEGFA) signaling is an important trigger of vascular sprouting, we next tested whether RIPK3 impacted VEGFA signaling in HUVECs. We found that RIPK3 knockdown surprisingly leads to

enhanced degradation of the vascular endothelial growth factor receptor VEGFR2 after stimulation with VEGFA. However, RIPK3 knockdown also paradoxically upregulates two key signaling pathways following VEGFA stimulation: ERK and Akt. Interestingly, a similar opposing effect on receptor degradation and signaling is also seen in RIPK3-depleted HUVECs following stimulation with three other vascular growth factors: epidermal growth factor (EGF), fibroblast growth factor (FGF), and transforming growth factor beta (TGFβ). Taken together, our data indicate that RIPK3plays a novel role in regulating receptor degradation and modulating ligand-induced signaling in ECs. Ongoing work is addressing the mechanism by which RIPK3 regulates these cellular processes and the in vivo implications for vascular sprouting and integrity.

P2336/B588

MAPK Feedback Phosphorylation of RGS Controls Its Spatiotemporal Localization and Alters Endocytosis through the Kelch Repeat Protein Kel1 during the Yeast Pheromone Response.

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G-protein coupled receptor (GPCR) signaling pathways regulate myriad cellular processes that require detection of an extracellular signal and conversion of that signal into a cytosolic response. The budding yeast, *Saccharomyces cerevisiae*, grows towards potential mating partners using a GPCR pathway to track the external gradient by initiating Rho-mediated cell polarization. This pathway makes use of two branches, a Gβγ branch that leads to Rho polarization and a MAPK cascade, and a less well understood Gα branch that anchors MAPK to the membrane to regulate formin activity. The mating pathway is desensitized by the regulator of G-protein signaling (RGS), Sst2. It has long been known that there is a MAPK feedback phosphorylation to the RGS, but this modification was found to have no effect on signaling from the Gβγ branch of the pathway. We hypothesized that the phosphorylation event serves to regulate the spatial and temporal dynamics of the RGS during the pheromone response to control where MAPK is active. Using a combination of live cell imaging in microfluidic devices and computational image analysis, we show that phosphorylation of the RGS results in loss of its localization to the site of polarization, and alters the pattern of active Gα-MAPK complex at the leading edge of the cell. In addition to the changes in localization of the RGS and its target Gα, we also came across two surprising results: 1) the ability to phosphorylate the RGS is required for the cell to successfully complete cytokinesis prior to beginning the mating response and 2) phosphorylated RGS leads to a decrease in endocytosis. These results lead us to consider the role of the formin regulator and member of the mitotic exit network, Kel1. Kel1 is a kelch repeat containing protein that has previously been found to interact with the RGS. Here we show that Kel1 is required for proper RGS localization, and provide evidence that RGS regulates Kel1 in a phosphorylation dependent manner. We propose that this newly discovered role for an RGS protein in the regulation of vesicle trafficking may function to fine tune the balance of endocytosis and exocytosis to promote the cell growth required for pheromone induced morphogenesis.

P2337/B589

Acetylcholine-induced Paneth Cell Granule Secretion Via Muscarinic M3 Receptor in Mouse Small Intestine.

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Background: Paneth cells at the base of small intestinal crypts secrete granules rich in antimicrobial α-defensin peptides and regulate the intestinal microbiota by killing pathogens. The involvement of dysbiosis, imbalance of the intestinal microbiota, in many diseases such as obesity and inflammatory bowel disease suggests the involvement of Paneth cell granule secretory responses in certain diseases. Since cholinergic carbamylcholine stimulates Paneth cell granule secretion, acetylcholine (ACh), an endogenous cholinergic neurotransmitter, may regulate the secretion. However, mechanisms including ACh receptor (AChR) expression and downstream signal cascades in Paneth cells remain largely unknown. **Objective:** We aimed to clarify mechanisms of Paneth cell granule secretion in response to ACh in cultured three-dimensional enteroids composed of small intestinal epithelial cells. **Methods:** Paneth cell granule secretion was visualized and quantified in mouse enteroids by granule area obtained by confocal microscopy before and after ACh stimulation. Functional muscarinic AChRs (mAChRs) and signaling was analyzed using antagonists selective for each mAChR subtype (M1-M5) and with inhibitors of protein kinase C (PKC) and inositol triphosphate receptor (IP3R). In addition, gene expression of each *mAChR in* Paneth cells was analyzed by quantitative PCR using singly-isolated Paneth cells. Ca^{2+} dynamics were analyzed by time-lapse Förster resonance energy transfer (FRET) imaging. To determine localization of submucosal cholinergic nerves, whole-mount immunostaining of small intestinal tissues against choline acetyltransferase (ChAT) and PGP 9.5 was performed, followed by tissue clearing. **Results:** Paneth cells secreted granules in response to ACh in a dose-dependent manner. Paneth cells expressed *M1*, *M3*, *M4*, *M5* genes but not *M2* among mAChR subtypes. Granule secretion was substantially inhibited only when enteroids were pretreated with M3 antagonist but not by others. Inhibitors of PKC and IP3R, the known major downstream molecules of M3 signaling significantly suppressed Paneth cell granule secretion. Rapid increase of intracellular Ca^{2+} concentration in Paneth cells was observed in response to ACh, followed by granule secretion. Immunostaining analysis further revealed that $PGP9.5^{+}$ $ChAT^{+}$ nerve endings were joined at the Paneth cell basement membrane, evidence that cholinergic nerve-derived ACh is involved in granule release. **Conclusion:** Paneth cells recognize ACh via the M3 receptor and secrete granules containing α-defensins via intracellular PKC and IP3R signals with increased cytosolic Ca^{2+} mobilization. This study contributes to understanding enteric pathophysiology related to disruption of Paneth cell.

P2338/B590

Protein Kinase a Inhibitor Protein (PKI) Levels Modulate ERK Activation Downstream of GPCR-cAMP Signaling.

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Protein kinase a (PKA)-inhibitor (PKI) proteins bind with high affinity to PKA and block its kinase activity, modulating the extent and duration of PKA-mediated signaling events. While PKA is a well-known regulator of physiological and oncogenic events, the influence of PKI proteins in these pathways has remained elusive. Here, by measuring activation of the MAPK pathway downstream of GPCR-Gαs-cAMP

signaling, we study how the expression levels of PKI protein can alter the balance of activation of two major cAMP targets: PKA and EPAC. Intracellular cAMP signaling mediated through two major effectors (PKA and EPAC) was modeled in HEK293 cells with over-expression or RNAi depletion of PKIs. Transcriptional programming alterations were determined with transcription factor-driven luciferase reporter assays. PKI expression was modulated to assess their role in switching signaling outcomes. Activating cAMP signaling downstream of Gαs with Forskolin (FSK) resulted in PKA-mediated inhibition of ERK phosphorylation. Over-expression of PKI and its subsequent inhibition of PKA rescued ERK activation. Utilizing a construct consisting of the PKA-inhibitory domain of PKI, these effects were confirmed to be due to specific inhibition of PKA signaling. Interestingly, activating mutations of Gαs and ligand stimulation of Gαs-coupled GPCRs resulted in slightly increased ERK activation; however, this activation was significantly enhanced with PKI over-expression. cAMP-mediated ERK signaling was found to be dependent on EPAC1-RAP1; where PKI could only partially rescue ERK activation in the absence of EPAC1. Modulation of PKI expression also significantly altered transcriptional programing downstream of the Gαs-cAMP signaling axis. Over-expression of PKI was shown to shift transcriptional activity away from PKA-CREB regulated genes towards those activated via ERK signaling. Together, these results indicate that PKI acts as a molecular switch driving cAMP signaling away from PKA and potentiating MAPK signaling.

P2339/B591

Single-molecule Imaging and an alysis of the Dynamic Organization of Vascular Endothelial Growth Factor 2 (VEGFR-2) on the Surface of Live Endothelial Cells.

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VEGFR2 is a receptor tyrosine kinase thatplays an essential role in promoting angiogenesis in response to its ligand VEGF. The canonical model has been that VEGF binds to VEGFR2 monomers, leading to VEGFR2 dimerization, autophosphorylation and downstream signaling. Recent studies suggest that VEGFR2 can dimerize in the absence of VEGF, and that VEGF affinity is higher to pre-dimerized VEGFR2. However, little is known about VEGFR2 spatiotemporal organization, including its oligomeric state, in its native plasma membrane environment in live endothelial cells. Therefore, we undertook a quantitative single-molecule study of VEGFR2 on the surface of live endothelial cells, labeling endogenous receptors and imaging the cell surface using TIRF microscopy, followed by automated single-molecule tracking and statistical data analysis. We found that VEGFR2 exhibits two movement types: (i) freely diffusing (~35%), with a mean diffusion coefficient of 0.18 um^2/sec; and (ii) confined/immobile (~65%). We observed a heterogeneity in VEGFR2's oligomeric state, with monomers, dimers and higher order oligomers/clusters. The freely diffusing molecules underwent more frequent interactions, but the confined/immobile molecules were on average of a higher oligomeric state. Addition of VEGF caused an almost immediate reduction in the diffusion coefficient of freely diffusing VEGFR2 molecules, followed by a more gradual shift (within 10 min) in the population from free to confined/immobile. Co-imaging of VEGFR2 and VEGF revealed that VEGF binds both monomeric and pre-oligomerized/clustered VEGFR2, with a preference for the latter, including those in the confined/immobile subpopulation. It also reduced the dissociation rate (to about one-half) of the dynamic interaction events. To understand better the link between VEGFR2 activation, its mobility and oligomeric state, we treated endothelial cells with the

VEGFR2 phosphorylation inhibitor AAL993. We found that AAL993 treatment increased the probability of free diffusion and the frequency of dynamic interactions both in the absence and presence of VEGF, suggesting that phosphorylation plays a critical role in the spatiotemporal organization of VEGFR2 at rest and upon ligand binding. Together, these observations depict a dynamic organization of VEGFR2 on the surface of endothelial cells that both affects and depends on VEGF binding and downstream activation.

P2340/B592

Prostaglandin E2 Induces Skin Aging by E-Prostanoid 1 receptor in Normal Human Dermal Fibroblasts.

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The production of collagen type I is decreased during aging, leading to skin wrinkle and impaired function. PGE2, a lipid derived signaling molecule derived from arachidonic acid by cyclo-oxygenase, inhibits collagen production and induces MMP1 expressions by fibroblasts in vitro. The inhibition of collagen expressions and the promotion of MMP1 by PGE2 have been accepted as the aging mechanisms. I demonstrated that EP1 plays an important role in PGE2 signaling in normal human dermal fibroblasts (NHDFs). When EP1 expression was suppressed with EP1 siRNA, there were no significant changes in mRNA levels of COL1A1/MMP1 between siRNA-transfected NHDFs and siRNA-transfected NHDFs with PGE2. These results show that EP1 is a receptor for PGE2. The phosphorylation of ERK1/2 after PGE2 treatment significantly increased by approximately 2.5 times. In addition, the exposure of NHDFs to PGE2 triggered an increase in intracellular Ca^{2+} concentrations. These results means that the PGE2 is directly associated with the EP1 receptor pathway-regulated ERK1/2 and IP_3 signaling in NHDFs. Acknowledgements: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (No. 201700280003).

P2341/B593

Molecular Mechanisms of STING Pathway Activation and Inhibition.

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The STING (STimulator of INterferon Genes) pathway is an innate immune signaling cascade which promotes essential anti-cancer, anti-viral, and anti-bacterial responses. Conversely, STING overactivation is linked to several autoimmune and inflammatory diseases such as Lupus, Multiple Sclerosis, heart attack, and Parkinson's disease. Despite its significance in the disease context, the precise molecular mechanism of STING activation and attenuation remains unclear. Using structural biology and biochemistry, we report that the metazoan second messenger 2'3'-cGAMP induces closing of the human STING homodimer and release of the STING C-terminal tail, which exposes a polymerization interface on the STING dimer and leads to the formation of disulfide-linked polymers via cysteine residue 148. Disease-causing hyperactive STING mutations either flank C148 and depend on disulfide formation or reside in the C-terminal tail binding site and cause constitutive C-terminal tail release and polymerization. Finally, bacterial cyclic-di-GMP induces an alternative active STING conformation, activates STING in a cooperative manner, and acts as a partial antagonist of 2'3'-cGAMP signaling. Our insights explain the tight control of STING signaling given varying background activation signals and provide a novel therapeutic hypothesis for autoimmune syndrome treatment.

P2342/B594

Biological Role of Oxytocin Receptor in Dental Pulp Stem Cells.

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Dental pulp stem cells (DPSCs) are mesenchymal stem cells in dental pulp, which induce the repair of damaged teeth. Oxytocin receptor (OXTR), a member of the G-protein coupled receptor, performs a variety of functions in living organisms such as smooth muscle contraction in lactation and delivery and various social behaviors including maternal behavior and pair bonds. Although many researches have been highlightedon the importance of OXTR, the biological function of OXTR in DPSCs is unknown. Here, we report the functional expression of OXTR in cultured human DPSCs (hDPSCs). Through genetic profiling and immunostaining procedures, we find that OXT and OXTR are expressed at high levels in human dental pulp and hDPSCs. Inhibition of OXTR increased the expression of osteo and odontogenic marker genes, promoting the hDPSC differentiation. The regulatory function of OXTR in the differentiation process is mediated by YAP, the downstream component of Hippo signaling pathway. Moreover, we find that OXTRs are involved in the extracellular matrix remodeling through regulating the expression of alpha-1 type I collagen (*COL1A1*) and matrix metalloproteinase-1 (*MMP1*) genes. These results demonstrate that OXTR mediates the intra- and extracellular functions of hDPSCs, indicating the biological importance of OXTR in pulp-dentin complex.

P2343/B595

The Splice Variants of gáQ Mediate Phototransduction, Rhodopsin Synthesis, and Retinal Degeneration in Drosophila.

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Heterotrimeric G proteins mediate a variety of signaling processes by coupling G protein-coupled receptors to intracellular effector molecules. In *Drosophila,* Gα49B gene encodes several Gqα splice variants and Gqα-PD isoform plays a predominant role in fly phototransduction. However, *Gqα-PD* null mutant flies still exhibit a residual light response, indicating other Gqα splice variants or additional G protein α subunits are involved in phototransduction. Here, we isolate a mutant fly with abolished light responses and the reduced rhodopsin level, and identify mutations in Gα49B gene and its two homologous gene, *CG30054* and *CG17760*. We demonstrate that Gqα splice variant Gqα4 mediates the residual light response in *Gqα-PD* null mutants. We further reveal that depletion of all Gqα splice variants leads to rapid light-dependent retinal degeneration, which is due to the formation of Rh1/Arr2 stable complexes. In addition, we reveal that Gqα homology CG30054 mediates rhodopsin synthesis whereas another Gqα homology CG17760 is expressed in pigment cells. Our study clarify the essential role of different Gqα splice variants in fly phototransduction and reveal the novel role of Gqα4 homology CG30054 in rhodopsin synthesis. Moreover, we demonstrate that completely depletion of Gqα protein also leads to rapid retinal degeneration.

P2344/B596

CXCR4 Localized at Intracellular Compartments Are Post-translationally Modified in Response to Plasma Membrane CXCR4 Activaton.

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The role of G protein-coupled receptor (GPCR) localization in regulating signaling has become increasingly apparent as distinct signaling events at the plasma membrane as well as endosomes and the Golgi have been shown. In addition, agonist-induced receptor post-translational modifications (PTM) have also been implicated in modulating both receptor regulation and signaling. Using wildtype and mutant CXC chemokine receptor 4 (CXCR4) that have distinct plasma membrane and intracellular compartment distribution, we investigated the role of receptor localization on agonist (CXCL12)-dependent CXCR4 PTM and signaling. Surprisingly, modulating CXCR4 plasma membrane localization did not change CXCL12-induced AKT phosphorylation or total receptor PTM. Furthermore, upon blocking receptor internalization, we found that both plasma membrane and internal pools of CXCR4 underwent CXCL12-dependent PTM. In contrast to previous reports where cell permeable agonist could potentially directly activate internal receptors, CXCL12 is membrane impermeable and, in accordance with our results, cannot directly activate CXCR4 at intracellular compartments. Receptor PTM is dependent on plasma membrane CXCR4 as removal of surface proteins abrogates CXCL12-induced CXCR4 PTM entirely. To investigate the functional significance of cells activating plasma membrane and internal pools of CXCR4, we monitored transcript levels of downstream CXCL12 target gene, EGR1. Surprisingly, cells with low plasma membrane CXCR4 expression exhibited significantly elevated agonist-induced EGR1 transcript levels compared to cells with high plasma membrane localization. Together these results suggest a model where plasma membrane receptors can communicate information to receptors at intracellular compartments upon ligand stimulation, by an unknown mechanism. Notably, CXCR4 activation of downstream transcriptional targets does not strictly correlate with CXCR4 plasma membrane expression, and this may have important implications when devising therapeutic strategies that consider both plasma membrane and internal pools of receptors.

P2345/B597

C-terminal Motifs Control Differential Desensitization, Internalization and Arrestin-coupling of Group II Metabotropic Glutamate Receptors.

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Precise regulation of glutamatergic signaling is imperative for normal brain function. In particular, metabotropic glutamate receptors (mGluRs) are G protein-coupled receptors (GPCRs) that fine-tune synaptic transmission by initiating intracellular signaling cascades that lead to short and long-term forms of plasticity. Of particular interest are mGluR2 and mGluR3, which have 70% sequence identity, both couple to the $G_{i/o}$ pathway, and are commonly known as presynaptic autoreceptors that inhibit glutamate release. Accordingly, precise regulation of mGluR2/3 signaling is important to preserve synaptic function. GPCR signaling is typically regulated by GPCR kinases which phosphorylate the C-terminal domain (CTD), leading to subsequent β-arrestin binding and endocytosis of the receptor to prevent excessive surface signaling. Studies aiming to define the differential regulation of mGluR2 and mGluR3 have been limited due to the lack of receptor subtype-specific drugs, antibodies, and methods with poor spatial resolution. We sought to determine whether mGluR2 and mGluR3 have distinct regulation by using a heterologous system to express SNAP-tagged mGluRs and specifically track the

surface receptor population. We found that unlike mGluR2, mGluR3 stably couples to β-arrestins and is endocytosed following activation. Further analysis of the CTD revealed that mGluR3 contains "phosphorylation codes" for optimal β-arrestin binding and inserting these codes into the CTD of mGluR2 allows it to stably couple to β-arrestins. Moreover, we discovered that a melanoma-associated mutation in a mGluR3 phosphorylation code enhances basal β-arrestin coupling and endocytosis. These results provide insight into the mechanism of β-arrestin coupling, and differential regulation between mGluR2 and mGluR3 suggests that they serve different functions at the synapse.

P2346/B598

Characterization of gβγ-pi3k-akt-mtor Signaling Pathway in the Calcium-sensing Receptor N639k Mutant Present in Breast Cancer.

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The calcium-sensing receptor (CaSR), a receptor coupled to G proteins (GPCR), physiologically regulates calcium homeostasis and of hormones, cytokines and growth factors secretion. This GPCR binds to three major groups of heterotrimeric G proteins: Gq/11, Gi/o and G12/13 and in cases such as cancer activates Gs. In addition, there is evidence that CaSR is capable of activating the PI3K-AKT-mTOR pathway through the βγ heterodimer and that this pathway regulates the secretion of certain chemotactic factors. It has been described that mutations in the intracellular loops produce an alteration in the coupling with the heterotrimeric G proteins having an impact on the signaling, however, there are few reports on the participation of the first intracellular loop with the coupling of the G proteins and its effect on the signaling. Considering that according to catalogue of somatic mutations in cancer (COSMIC) database CaSR is the second GPCR most mutated in cancer, we decided to study CaSR mutations in breast cancer. Preliminary results show that $CaSR_{N639K}$ mutant has a switch in the coupling of Gα proteins. In this work, we decided to further characterize the effect of the N639K mutant, located in the first intracellular loop of the CaSR, in the signaling pathway of Gβγ-PI3K-AKT-mTOR to examine the effect of Wortmannin, a PI3K inhibitor and Rapamycin, an inhibitor of the mTOR complex in the activation of AKT, S6 and its impact on chemotactic factors secretion. Results obtained show that $CaSR_{N639K}$ mutant has a reduced capacity, with respect to the $CaSR_{WT}$, to promote the signaling of the Gβγ-PI3K-AKT-mTOR pathway in response to extracellular calcium and the calcimimetic R-568. In addition, this effect correlates with a minor on chemotactic factors secretion, detected in conditioned media, specifically interleukin 8 (IL-8) and monocyte chemoattractant protein-1 (CCL2 / MCP-1), which were identified by flow cytometry. Our results suggest that the $CaSR_{N639K}$ mutant localized in the first intracellular loop produces an inactivating effect of Gβγ-PI3K-AKT-mTOR signaling pathway, affecting the secretion of chemotactic factors, which might also suggest that the first intracellular loop could be participating in the coupling with heterotrimeric G proteins.

P2347/B599

Effects of Tbbpa on Growth Factor Receptor Tyrosine Kinase Markers in a Human Uterine Endometrial 3d Culture Model.

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Tetrabromobisphenol a (TBBPA) is a brominated flame retardant (BFR) widely used world-wide. It is ubiquitous in the environment and its exposures have led to detectable levels in humantissues. TBBPA is an endocrine-disrupting compound (EDC) and may mimic physiologic estrogen, or disrupt estrogen homeostasis resulting in excess estrogen levels. Due to TBBPA's estrogenic properties and its ability to induce endometrial cancer in female Wistar Han rats in a National Toxicology Program (NTP) 2-year bioassay, we were interested in evaluating its effects on human endometrial spheroids in culture. Additionally, in rats that had in *utero* and early-life exposures to TBBPA, we found increased uterine expression of growth factor receptor tyrosine kinases (RTKs) similar to those overexpressed in endometrial cancer in women, which is the seventh most common type of cancer in women. In this study, the human uterine endometrial epithelial cell (Ut-EEC), that was found to be a target cell type of cancer development in the NTP TBBPA rat bioassay was used to create a 3D culture system. Prior to formation of 3D cultures, 2D cells were characterized by immunocytochemistry for the epithelial cell markers, anti-pan cytokeratin and anti-epithelial antigen, and by "DNA fingerprinting" to determine the cultures were HeLa cell negative. We observed increased proliferation at low concentrations of TBBPA (10^{-6} µM - 1 µM), whereas higher concentrations (10 µM - 200 µM) decreased growth in 2D cultures of ht-EEC cells. The cells in 3D culture were treated with TBBPA at an concentration of 10^{-3} µM. The RTK arrays revealed TBBPA at 10^{-3} µM upregulated the expression of several activated RTKs, including EGFR, Insulin R and IGF-1R that have been reported to be biological markers of endometrial cancer in women. These data are consistent with what we have found in TBBPA in *utero* and perinatal rat studies, and provide evidence of TBBPA at human-relevant exposure levels may pose a potential hazard for human health, particularly in women. Therefore, this study not only provides a possible mechanism of TBBPA effects on human uterine endometrial cells, but also provides an appropriate 3D in *vitro* model for studying environmental toxicants that target endometrial epithelial cells.

P2348/B600

Appl1 and Akt2 Mediate Non-genomic Progesterone Signaling.

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The steroid hormone progesterone (P4) regulates many critical aspects of vertebrate reproductive and general physiology. P4 signaling is mediated through its nuclear receptors to modulate gene expression, or on a more rapid time scale through the so-called non-genomic signaling mediated by membrane P4 receptors (mPRS): α/β/γ/δ/ϵ, among other effectors. Signaling downstream of mPRs remains poorly understood. Here we show that the topology of mPRβ is opposite to that of G-protein coupled receptors (GPCR) with a cytosolic N-terminus and an extracellular C-terminus. Using *Xenopus* oocyte maturation as a well-established physiological readout of non-genomic P4 signaling, we found that mPRβ signaling requires APPL1 (Adaptor Protein containing Pleckstrin homology domain Phosphotyrosine binding domain and Leucine zipper motif 1) and the kinase AKT2. We further show that mPRβ non-genomic signaling requires clathrin-dependent endocytosis that is APPL1 dependent, resulting in a spatially

transient co-localization of mPRβ with the signaling partners APPL1 and AKT2 to mediate downstream signaling. We extended the *Xenopus* oocytes results to mammalian cells and found that APPL1 mediates P4-non genomic signaling in the Glutag enteroendocrine cells. Our findings are first to outline the early steps involved in mPRs non-genomic signaling.

Signaling Scaffolds and Microdomains

P2349/B601

The Role of Membrane Lipids on T Cell Antigen Receptor Triggering.

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T cell antigen receptor (TCR) recognizes antigenic peptide conjugated major histocompatibility complexes (pMHC) to trigger T cell activation and induce immune response against invading bactrium or virus. The αβ TCR consists of a variable heterodimer that associates noncovalently with the CD3 and ζζ dimer to form a multisubunit complex across the membrane. Both TCR subunits do not contain any signaling module, its signaling activity depends on CD3s. Upon antigen recognition, the cytoplasmic domain of CD3s will be phosphorylated by Src-family kinase Lck to transduce the signal downstream. Yet how TCRs transmit the antigen recognition signal into the cell across the cell membrane remains elusive. Previous studies suggest that TCRs function as a force sensor, thus it is straight forward to propose that mechanical force may facilitate TCRs' transmembrane signaling. Here by combining molecular dynamics simulation, biochemical and cell biology studies, we found that mechanical force deforms cell membrane which directly leads to membrane reorganization. The reorganized membrane forms microdomain which help to activate both Lck kinase and the CD3 intracellular domains for effective phosphorylation on the various immunoreceptor tyrosine-based activation motifs (ITAM) of CD3s.

P2350/B602

Tetraspanin Scaffold Regulation of Epidermal Growth Factor Receptor Organization and Signaling.

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Molecular interactions on the plasma membrane can significantly influence the outcomes of signaling events crucial to cell function in both normal and disease states. Tetraspanins, a superfamily of transmembrane proteins, play a vital role as scaffolding proteins on the membrane, forming structural platforms termed Tetraspanin-Enriched Microdomains (TEMs). By facilitating compartmentalization, tetraspanins have been shown to act as regulators of cellular signaling, enhancing membrane recruitment of signaling proteins like PKCα, and altering the organization of adhesion molecules like integrin α4. Here, we assess the role of tetraspanin CD82 in the regulation of Epidermal Growth Factor Receptor (EGFR) signaling. Signaling by EGFR, a receptor tyrosine kinase, has been shown to depend on dimerization of receptors and subsequent internalization, processes that are subject to the dynamics of the plasma membrane. Disruption of EGFR signaling has been implicated in many disease states, including various cancers. Genetic and biochemical studies have previously shown that specific tetraspanins can be found to directly and indirectly influence EGFR signaling and internalization. However, the mechanisms by which tetraspanins modulate EGFR signaling are not fully understood. Using direct stochastic optical reconstruction microscopy (dSTORM) to achieve sub-diffraction limit resolution, we mapped the relative organization of EGFR with tetraspanin CD82 on the plasma

membrane of HeLa and HEK293 cells. Pair cross-correlation analysis of the distribution of these molecules reveals a positive association between EGFR and CD82, while clustering analyses show a re-organization of CD82 following EGF treatment. To further analyze the impact of CD82 on the function of EGFR, we generated cell lines overexpressing either CD82 or a CD82 palmitoylation mutant and measured EGFR phosphorylation following EGF stimulation. We find that while CD82 overexpression increases basal EGFR activation, signaling is significantly attenuated following stimulation. Moreover, our data suggests that the attenuation of EGFR signaling by CD82 involves integrin engagement with extracellular matrix. Collectively, these studies suggest that the CD82 scaffold directly interacts with EGFR to regulate signal transduction. Future studies will be directed towards understanding the contribution of tetraspanin-tetraspanin and tetraspanin-cholesterol interactions in the regulation of EGFR signaling and dynamics.

P2351/B603

Nik-related Kinase Suppresses Cell Proliferation by Modulating CK2-PTEN-Akt Signaling Pathway.

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Nik-related kinase (Nrk) is one of Germinal Center Kinase family proteins. Nrk expression is restricted to a few tissues including placenta and mammary gland. We had shown that Nrk plays important roles in the prevention of the development of placental hyperplasia and breast cancer. In this study, we investigated the mechanisms by which Nrk suppresses cell proliferation. Nrk harbors a kinase domain at the N-terminal region and uncharacterized CNH domain at the C-terminal region. We generated HEK293 cells with the doxycycline-inducible expression of Nrk or the mutants. The expression of wild-type Nrk suppressed cell proliferation and decreased Akt phosphorylation without affecting Erk phosphorylation. The expression of kinase-dead Nrk or a truncated form of Nrk lacking the kinase domain also suppressed cell proliferation and decreased Akt phosphorylation, indicating a kinase-independent action of Nrk. Neither truncated forms of Nrk lacking the middle region nor the CNH domain affected cell proliferation and Akt phosphorylation, indicating the requirement of these regions for Nrk action. Immunostaining analyses revealed that wild-type Nrk is localized at the plasma membrane in HeLa cells, and the CNH domain is required for the localization. To elucidate detailed mechanisms by which Nrk inhibits Akt signaling at the plasma membrane in a kinase-independent manner, we tried to identify Nrk-interacting proteins. Co-immunoprecipitation and LC-MS analyses revealed that Nrk interacts with casein kinase-2 (CK2). We confirmed that Nrk interacts with CK2 beta subunit by co-immunoprecipitation and immunoblotting, and found that the middle region of Nrk is required for the interaction. It is known that CK2 phosphorylates C-terminal sites of PTEN and inhibits PTEN activity, leading to the accumulation of PIP3 followed by enhanced Akt signaling. We found that doxycycline-induced expression of wild-type Nrk in HEK293 cells decreased PTEN phosphorylation at the C-terminal site as well as Akt phosphorylation, suggesting PTEN activation. The treatment with a PTEN inhibitor VO-OHpic restored Akt phosphorylation in Nrk-expressing cells, not in control cells. Taking these results together, we propose the following model by which Nrk suppresses cell proliferation: 1) Nrk is localized at the plasma membrane via the CNH domain; 2) Nrk interacts with CK2 via the middle region, and possibly inhibits CK2 activity; 3) Nrk thereby modulates CK2-PTEN-Akt signaling pathway at the plasma membrane and decreases Akt-mediated proliferative signaling, leading to the suppressed cell proliferation. Possibly through these mechanisms, Nrk prevents the development of placental hyperplasia and breast cancer.

P2352/B604

IQGAP1 Scaffolding Connects EGFR and Phosphoinositide Signaling to Cytoskeletal Reorganization.

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IQGAP1 is a multi-domain protein that acts as a scaffold for multiple signaling pathways, especially in cancer cells where it is seen to be overexpressed. While IQGAP1 is known to generate the lipid messenger PI(3,4,5)P_3 by scaffolding the phosphoinositide kinases PIPKIs and PI3K, the dynamics of this scaffold in intact, living cells remains unknown. Here, we delineate the spatial and temporal effects of IQGAP1 signaling in live cells under basal and stimulated conditions using fluorescence lifetime imaging microscopy and other biophysical techniques. We observe EGF-mediated changes in both IQGAP1 and EGFR distribution and see that IQGAP1 is involved in EGFR clustering. We also demonstrate that IQGAP1 interacts strongly with PIPKIγ at intracellular compartments and at the plasma membrane, and that it scaffolds PI3K and PIPKIγ in response to certain physiological changes. Additionally, we show that IQGAP1 scaffolds phosphoinositides with PI3K, PIPKIγ and EGFR, and forms clusters upon cell stimulation with EGF. Importantly, we show that IQGAP1 connects PI(3,4,5)P_3-mediated signaling and cytoskeletal signaling pathways by binding PIPKIγ in proximity of the cytoskeletal proteins talin and Cdc42. Our results support a model in which IQGAP1 mediates crosstalk between EGFR receptor activation, phosphoinositide signaling and the cytoskeleton.

P2353/B605

Localized Intercellular Transfer of Ephrin-As by Trans Endocytosis Provides a Memory of Signaling.

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Alterations in different biosynthetic trafficking routes lead to losses in cellular polarity, which represent the earliest stages of carcinogenesis. We focused on the trafficking pathways of the tyrosine kinase receptor EphA2 and its membrane-bound ligand ephrinA1. EphA2 is frequently overexpressed in melanoma, gliomas, and around 10 different types of carcinomas where its levels are associated with poor prognosis. The colocalization of EphA2 with the basolateral protein E-cadherin and the presence of an usually apical sorting motif (GPI anchor) in ephrinA1 strongly suggest that both proteins are segregated from each other in their journey to the plasma membrane. To be able to study specifically, quantitatively and in real-time specific trafficking pathways our laboratory has developed a system called RUSH (Retention Using Selective Hooks), which allows to synchronize the secretion of virtually any protein. By using the RUSH system, we demonstrated that EphA2 and ephrinA1 have different kinetics of transport and are partially sorted from each other at the level of the Golgi apparatus. Upon arrival to the plasma membrane, we observed that ephrin A1/EphA2 complexes are locally formed at the tip of filopodia, at cell-to-cell contacts. Clusters of ephrin A1 from donor cells surf on filopodia associated to EphA2-bearing sub-domains of acceptor cells. Full-length ephrin A1 is transferred to acceptor cells by trans endocytosis through a proteolysis-independent mechanism. Trans-endocytosed ephrin A1 bound to its receptor enables signaling to be emitted from endo-lysosomes of acceptor cells. Localized trans-endocytosis of ephrin-A1 sustains contact-mediated repulsion on cancer cells. Our results uncover the essential role played by local concentration at the tip of filopodia and the trans-endocytosis of full-length ephrin to maintain long-lasting ephrin signaling.

P2354/B606

Cd47 Positions Sirpa to Prevent Integrin Activation and Engulfment.

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Macrophages must engulf dead cells, debris, and pathogens, while selecting against healthy cells to prevent autoimmunity. Healthy cells express CD47 on their surface, which activates the SIRPA receptor on macrophages to suppress engulfment. Cancer cells overexpress CD47 to evade clearance by the innate immune system, making the CD47-SIRPA signaling axis an appealing therapeutic target. However, the mechanism by which CD47-SIRPA inhibits engulfment remains poorly understood. Here, we dissect SIRPA signaling using a reconstituted target with varying concentrations of activating and inhibitor ligands. We find that SIRPA is excluded from the phagocytic synapse between the macrophage and its target unless CD47 is present. Artificially directing SIRPA to the kinase-rich synapse in the absence of CD47 activates SIRPA and suppresses engulfment, indicating that the localization of the receptor is critical for inhibitory signaling. CD47-SIRPA inhibits integrin activation in the macrophage, reducing macrophage-target contact and suppressing phagocytosis. Chemical activation of integrins can override this effect and drive engulfment of CD47-positive targets, including cancer cells. These results suggest new strategies for overcoming CD47-SIRPA inhibition of phagocytosis with potential applications in cancer immunotherapy.

P2355/B607

The Role of Non-motif Selectivity Determinants in Peptide-binding Domain Interactions.

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An important class of protein-protein interactions in the cell involve recognition of short linear motifs (SLiMs) or peptides which often have a relatively weak affinity and are transient. Dysregulation of SLiM-binding domains and target interactions are implicated in a number of human diseases. Due to the recognition of only a couple of amino acid positions by SLiM-binding domains, interaction networks overlap and specific targeting is hard to achieve. The PDZ domain was used as a model system in order to define the role of non-motif selectivity determinants in SLiM- or peptide-mediated interactions. They recognize the extreme C-terminus of target proteins and binding motifs are based on only two residues. Dissecting the binding networks of over 250 PDZ domains using only motif preferences is impossible. Likewise, two sequences can bind drastically different numbers of PDZ domains. For example, the C-terminal sequence of the human papillomavirus E6 oncoprotein (HPV16 E6) interacts with over a dozen PDZ domain-containing proteins, while the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR) interacts with less than five, yet both contain identical motif residues. We reveal that single-residue substitution of peptides and structural biology allow us to determine non-motif selectivity determinants for multiple PDZ domains that target the HPV16 E6 oncoprotein. In addition, we present the first known crystal structure of a PDZ domain from choanoflagellates, our closest non-metazoan ancestor, and describe non-motif selectivity determinants in these unique organisms. We show that non-motif specificity is critical in order to characterize SLiM interactions networks in the cell.

P2356/B608

Syndecan-4 Proteoglycan Regulates Caveolin1 Expression in Endothelial Cells.

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Syndecan-4 (Syn4) is a transmembrane heparan sulfate proteoglycan that has been shown to regulate focal adhesions and to play a crucial signaling center for proper communication between intracellular, cell-surface and cell-extracellular matrix. Normal vascular endothelial cells (EC) from rabbit aorta and Syn4 knockdown cells (shRNA-Syn4-EC) were transfected with vectors encoding caveolin1 targeted with mCherry fluorescent protein (CAV1-mCherry) and clathrin light chain targeted with mRFP fluorescent protein (CLC-mRFP). Cells were cultured in glass coverslips (12 mm, 3 days) in F12 medium supplemented with 10% fetal serum bovine, streptomycin and penicillin (both 100 IU/mL) at 37ºC in a humidified atmosphere (2.5% of CO_2). DAPI was used to stain nucleus. Images were captured to in *vivo* cells with a confocal scanning microscope equipped with a Plan-Apochoromat x63 objective (numerical aperture 1.4) under oil immersion. Heparitinase I and heparitinase II were heterologous produced. CAV1-mCherry and CLC-mRFP cells were treated with heparitinase I and II for 30 minutes and images were taken every 2 minutes. Total RNA were extracted from EC and shRNA-Syn4-EC cells and both semi-quantitative (RT) and quantitative (q; real-time) polymerase chain reaction (PCR) were performed. Total proteins were extracted from EC and shRNA-Syn4-EC cells and western blot were performed. Our results show that lack of Syn4 decreases Caveolin1 gene and protein expression. CAV1-mCherry cells treated with heparitinase I and II change Caveolin1 clusters dynamics in 10 minutes. Syn4 knockdown also induces C-myc, C-myb and Ebf2 transcription factor expression and downregulates Gata3 and microRNA204 expression. The gathered data shows the pivotal role of Syn4 in cell signal transduction and further pinpoint its importance for Caveolae clusters dynamics.

P2357/B609

Ultrafine Carbon Black Promotes Lysosomal Membrane Permeabilization and Nlrp3 Inflammasome Activation.

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Carbon black (CB) particulates are a central component of diesel exhaust, and a major airborne pollutant of ultrafine particulate matter. CB exposure contributes to the adverse health effects of pollutants upon the lungs, heart, and nervous system. A primary source of damage is the chronic inflammation induced by exposure to airborne particulates. To examine the capacity for ultrafine CB (~30-130 nm in diameter) to accumulate in macrophage and to stimulate pro-inflammatory signaling, RAW264.7 mouse macrophage and THP-1 human monocytes were used. CB particles were hypothesized to enter immune cells by endocytosis where they accumulate, and larger particles are visible within vesicles and cytosol. The accumulation of CB in RAW264.7 cells was evaluated over a 36-hour period. At specific time points, cells were washed extensively and then lysed to release cellular components. Accumulated particulates were purified from lysates via centrifugation, and then quantified via absorbance readings (A595). Particles were shown to accumulate gradually over time, with significant accumulation being detected after 2 hours of exposure with doses of 25, 50 and 100 ug/ml. Additional studies used THP-1 monocytes to evaluate inflammasome signaling. THP-1 monocytes were stimulated with phorbol-12-13-acetate (PMA) to induce differentiation and adherence. To evaluate the hypothesis that CB accumulation impacts membrane integrity in lysosomes and phagolysosomes we assessed cathepsin B release into the

cytosol and Galectin-3 localization to lysosomal vesicles. Each of these assays provided support for a model of CB-induced lysosomal membrane permeabilization (LMP). In addition to LMP, we hypothesized that CB could stimulate the assembly of the inflammasome and a subsequent production and release of IL-1β. Differentiated THP-1 cells were stimulated with lipopolysaccharide (100ng/ml) to upregulate expression of inflammasome components such as NLRP3 and ASC proteins. Assessment of inflammasome assembly and activity was determined by measuring ASC oligomerization, formation of cytosolic ASC "specks", and accumulation of IL-1β in the extracellular environment. An alysis of ASC oligomerization as well as fluorescent tagging of ASC oligomer complexes (specks) provided some evidence for CB activation of the inflammasome, though a clear potential for LPS to similarly stimulate the inflammasome was observed. Further evaluation of these conditions and results will be discussed at the poster session. The future direction of this work will focus upon upstream mechanisms of inflammasome signaling that are affected by CB accumulation.

Mechanotransduction 2

P2358/B611

Mechanically Driven Cellular Competition Promotes the Collective Extrusion of Bacteria-infected Epithelial Cells.

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Intracellular bacterial pathogens often alter the behavior of their host cells, including their mechanics, to promote their own survival and spread. For instance, food-borne *Listeria monocytogenes* secretes effector proteins that loosen the cortical tension of infected epithelial cells facilitating its spread from cell to cell or through basement membranes. What is less clear is whether host cells are capable of changing their biomechanics in response to infection in a way that would benefit the host by limiting bacterial spread. Inspired by this question, we followed Madin-Darby Canine Kidney epithelial cell monolayers infected with low levels of *L. monocytogenes*over the course of 3 days. We found that, as the bacteria replicate and spread intercellularly, the host cells containing bacteria get progressively squeezed by surrounding uninfected cells. Surrounding uninfected cells become highly migratory, elongate perpendicular to the tangent of the focus, and squeeze the infected cells that eventually get extruded. Extruded cells continue adhering to the cellular monolayer giving rise to "mounds" of infected cells. We hypothesized that cellular compression and extrusion of infected cells is driven by changes in the mechanics of uninfected and infected cells. Indeed, when we measured the traction forces that cells exert while residing on deformable matrices mimicking their natural environment, we found that infected cells weaken their cell-substrate adhesion-dependent forces over time as compared to nearby uninfected cells. Concurrently, infected cells get softer than surrounding uninfected cells, and collectively the monolayer behaves more like a fluid with a higher diffusion coefficient as compared to more solid-like cells from an uninfected monolayer, a behavior reminiscent of the jamming-unjamming transition. We are using 3D agent-based modeling to model infection-driven cellular extrusion in order to understand how changes in cellular stiffness, cell-matrix and cell-cell forces alone and synergistically can drive the observed cellular competition between infected and uninfected cells. The goal is to uncover how the mechanical forcesthat lead tothe creation of extrusion domains of infected cells are coordinated with the underlying biochemical changes associated with infection, and in particular with

innate immune defense pathways and NF-kB activation. Overall, our findings underline the dynamic remodeling capability of epithelial tissue and might hint at a potential mechanism employed by host epithelial monolayers to contribute to elimination of infected host cells.

P2359/B612

Hydrostatic Pressure Gradient Causes Spatio-Temporal Calcium Oscillations and Alters Apico-Basal Polarity in Renal Epithelial Cells.

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Using a novel microfluidic platform to recapitulate fluid transport activity of epithelial cells, we report that MDCK-II and human primary kidney epithelial cells can actively generate hydraulic pressure gradients across the epithelium. The fluidic flux declines with increasing hydraulic pressure until a stall pressure, at which the fluidic flux vanishes--in a manner similar to mechanical fluidic pumps. The developed pressure gradient translates to a force of 50-100 nanoNewtons per cell. For MDCK-II epithelium, basolateral hydrostatic pressure gradients lead to high frequency spatial and temporal calcium oscillations. Extracellular calcium chelation and inhibition of mechanosensitive ion channels indicate that local stretch in the plasma membrane due to fluid pressure caused intracellular calcium enrichment. Hydrostatic pressure also caused cell height to double within 15 minutes and subsequently recovered in 60 minutes. Change in cell height was coupled with high frequency plasma membrane invaginations, rapid F-actin turnover and decreased Na^+/K^+ ATPase expression on the basolateral domain. These results, together with data from osmotic and pharmacological perturbations of fluidic pumping, implicate a novel mechanism of mechanosensation, and provide further insights into the regulation of fluid transport in epithelial cell.

P2360/B613

Epithelial Tissue Geometry Directs Emergence of the Bioelectric Field and Patterns of Growth.

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Cellular membrane voltage (Vm), defined as the electric potential difference between the cytoplasm and extracellular medium, forms gradients of bioelectricity across tissues. Manipulating Vm and the resulting bioelectric field powerfully influences proliferation, regeneration, and tumorigenesis at the tissue-scale. However, it remains unclear how Vm is coordinated to produce bioelectric gradients across tissues, or whether Vm is impacted by other factors in the cellular microenvironment, such as mechanical stress. Here, we used micropatterned epithelial tissues to investigate the relationship between tissue geometry, mechanical stress, and patterns of Vm. We found that cells located in high-stress regions of mammary epithelial tissues are more positively charged, or depolarized than those in low stress regions. This pattern of Vm correlates with the pattern of proliferation in the tissue. Inhibiting mechanical force transmission by deleting or mutating E-cadherin abolished the patterns of Vm and proliferation gradients within the tissues. These data suggest a role of mechanical force in the emergence of bioelectric fields. We further determined that intracellular Ca^{2+} is required for regulation of Vm by tissue geometry, implying a connection between bioelectricity and proliferation via calcium signaling in the context of mechanical force. Our data suggest that tissue geometry controls bioelectric gradients and proliferation through patterns of mechanical stresses.

P2361/B614

Role of Linker of Nucleoskeleton and Cytoskeleton (LINC) Complex in Endothelium Homeostasis.

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The linker of nucleoskeleton and cytoskeleton (LINC) complex is localized in nuclear membrane and plays a central actor for forces transmission between nucleus and cytoskeleton. The LINC complex is composed by two major components, SUN proteins and KASH proteins, that making the link between nucleoskeleton and cytoskeleton respectively. In different cellular contexts, this complex is known to play a crucial role in cellular organization, cell polarity, cellular motility and DNA stability.We hypothesized that the LINC complex is important in the physiology of vascular cells. Endothelial cells, as the inner most cell layer in blood vessels, are subject of different mechanical stresses imparted by blood flow, as stretch and pressure. In this study, we investigate the importance of the LINC complex, using dominant negative KASH (DN-KASH) to disrupt the LINC complex in human umbilical vein endothelial cells (HUVEC). Expression of DN-KASH expression alters intercellular junction establishment, as well as the responses of cell-cell contacts to cytokine challenge. In addition, disruption of the LINC complex induces cell detachment under arterial levels of fluid shear stress. Taken together our results show that the LINC complex is necessary for endothelial stability, including cell-cell and cell-matrix adhesions. We are currently investigating the hypothesis that a cytoskeleton-nucleoskeleton disconnection induces an aberrant forces distribution in endothelial cells by a loss of mechanical “feed-back”, leading to an alteration of endothelium stability and associated functions.

P2362/B615

How Adherent Cells Reorient in Response to Cyclic Stretching.

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Mechanical stimulation by forces transmitted through the surrounding solid and fluid materials plays an important role in regulating the function of many tissues from the subcellular to organ level. Intriguingly, while cells often respond to non-cyclic mechanical cues along the direction of stimulation, adherent cells often reorient perpendicularly to the direction of uniaxial cyclic stretching. While this phenomenon has been documented for decades, the underlying control mechanism remains poorly understood. To address this question, we have developed a simple on-stage stretching approach that allows programmable stretching-relaxation and synchronized imaging of cells cultured on polyacrylamide substrates. We observed that the reorientation of NRK epithelial cells involves shortening along the direction of stretching (referred to as longitudinal) and extension perpendicularly to the direction stretching (referred to lateral). Using substrates with micropatterned lines to constrain cell shape, we found that these responses are independent of each other such that lateral elongation may take place without longitudinal shortening, and vice versa. By analyzing differences of images collected at the beginning and end of stretching or relaxation phases, we show that lateral elongation takes place predominantly during the relaxation phase, indicative of a global shortening signal that takes effect during both stretching and relaxation phases in conjunction with a lateral elongation signal that takes effect only during the relaxation phase. By systematically varying the duration of stretching or relaxation phases while analyzing the cell orientation parameter, $\cos 2\theta$, we show that optimal lateral elongation requires a specific duration of stretching such that the effect diminishes upon prolonged stretching phase. Similarly, at a given length of stretching, the effect on lateral elongation dissipates upon

prolonged relaxation. Together, these results suggest that stretching generates a transient global retraction signal that takes effect right away, and a latent lateral elongation signal that primarily takes effect during the following relaxation phase. In conclusion, the net effect of reorientation is determined by the interplay of these retraction and elongation signals and their respective kinetics of generation and degradation, in conjunction with the adaptation of cells to a stretched substrate through the formation of new adhesion structures.

P2363/B616

Cellular Realignment Upon Applied Stress in Three-dimensional Microtissues.

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Multicellular tissue self-assembly at the cell-cell and cell-matrix levels is governed by chemical and mechanical cues. A major mechanical cue that controls tissue integrity and is a probable inducer of cell alignment in processes such as vasculogenesis is stress stimulation. To study the role of stress stimulation, high-throughput mechanical conditioning assays have been developed for systems ranging from centimeter-scale to submillimeter-scale tissues. However, currently these systems are limited to applying uniaxial stresses. To address this limitation, we designed and fabricated a high-throughput and easy-to-implement pneumatically-controlled microfluidic module integrated with square-shaped microtissues formed with 3T3 fibroblasts in type I collagen adapted from Legant, W. R. et al. Proc. Natl Acad. Sci. 2009. This microfluidic system has wells underneath the pillars supporting the tissues and as vacuum is applied, the wells in the device evacuate and the thin membrane between the wells and pillars deflects, stretching the microtissue seeded on it. The placement of the wells controls the direction of the stress, allowing induction of stress in multiple directions. Using this device, we investigate the role stress stimulation plays in cell realignment. We quantify the coarse-grained cell orientation field using the nematic order parameter $Q = (\langle \cos 2\theta \rangle^2 + (\langle \sin 2\theta \rangle^2)^{1/2}$ with Q ranging from 0 to 1 where Q=1 is perfect alignment. Under no stress, square tissues have disordered cells in the center due to the isotropic arrangement of the pillars. Upon cyclic stretch, we observe high cell alignment in the center with $Q = 0.87 \pm 0.02$ as compared to the non-stretched control tissues of $Q = 0.49 \pm 0.04$. Our microfluidic platform provides a robust method to induce stress stimulation in three-dimensional microtissues and permits quantitative studies of fundamental cellular mechanics.

P2364/B617

Spatiotemporally Distinct Cell Behaviors Drive Multilayered Tissue Spreading.

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Spreading of embryonic tissue plays a critical role to construct various shapes of body parts during development, yet little is known about the cellular mechanisms that drive multilayered tissue spreading. Here we take advantage of the multilayered ectoderm tissue isolated from an early stage of Xenopus embryo that autonomously spreads out on the fibronectin-coated substrate similar to in vivo. Quantification of tissue spreading using particle image velocimetry (PIV) indicates the emergence of directed tissue movements along the edge at around 6 hours after spreading which is coincide with elevated nuclear YAP localization. We begin to dissect the role of cell behaviors including directed cell movement, cell division, and radial intercalation that can contribute to the mechanically distinct phase

of tissue spreading. Quantifying tissue spreading ratio, cell displacements, spatiotemporal patterns of cell behaviors combined with altered F-actin dynamics, cell divisions, and intercalations propose distinct contributions of cell behaviors at different stages of multilayered tissue spreading. Our integrative analysis of spatiotemporally distinct cell behaviors during multilayered tissue spreading will provide fundamental insight for morphogenesis, disease progression, and tissue engineering.

P2365/B618

Tissue Mechanics in Initiation and Propagation of Epithelialization on the Surface of Deep Ectoderm Aggregates.

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Cells in our body are highly plastic which can switch their phenotypes in the range from mesenchymal to epithelial as they needed. Our previous study shows that tissue mechanical property and cell contractility play a critical role for epithelialization on the surface of deep ectoderm aggregates generated from early Xenopus laevis embryo. However, it is still vague how local tissue mechanics may direct the initiation and propagation of epithelialization to cover the entire surface of the embryonic aggregates. Using RFP-ZO1 we follow the spatiotemporal dynamics of epithelialization on the surface of the deep ectoderm aggregate and begin to find single or patches of epithelial cells emerge from 2 hours-post-aggregation (hpa). Newly epithelialized cells often laterally added to existing epithelial cells. Groups of epithelial cells merge and expand to cover the aggregate surface. To understand the role of local cell contractility to initiate and propagate the observed epithelialization, we adopted optogenetics using Cry2-RhoA. With blue light Cry2-RhoA clustered and located to the membrane that consequently induce cell contractility and shrinkage of the cell size by ~15%. This in *vitro* system of live embryonic aggregates that can regenerate new epithelium combined with optogenetics provides a platform to interrogate the correlation between local tissue mechanics and epithelialization.

P2366/B619

Substratum Stiffness Regulates Erk Signaling Dynamics in Mammary Epithelial Tissues.

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The Ras/Erk signaling cascade is critical for tissue development and homeostasis, and coordinates the growth, proliferation, and migration of cells. Aberrations in Erk signaling result in developmental disorders and promote uncontrolled cell growth and proliferation, which are hallmarks of cancer. Efforts to understand the dynamics of Erk signaling have largely focused on how growth factor stimulation influences the dynamic output of Erk activity. However, Erk dynamics also appear to be influenced by mechanical stimuli. We investigated the influence of substratum stiffness on Erk dynamics by culturing mammary epithelial cells on substrata of different stiffnesses and monitoring Erk dynamics at the single-cell level using a kinase translocation reporter. Cells cultured under high growth factor concentrations exhibited pulsatile Erk dynamics whose amplitude and frequency increased as substratum stiffness increased. Similarly, stimulating growth factor-starved cells with epidermal growth factor (EGF) on soft substrata resulted in a low-amplitude, transient Erk activation, in contrast to the high-amplitude, sustained Erk activation in cells on stiffer substrata. Using optogenetics to acutely stimulate Ras produced a sustained Erk response irrespective of substratum stiffness, suggesting that the mechanical microenvironment regulates Erk signal transmission at nodes upstream of Ras. Consistently, we found that EGFR expression is significantly decreased in cells cultured on soft substrata, which might account

for the increases in EGFR phosphorylation and downstream Erk activation in cells on stiff substrata. Taken together, our work establishes a framework for how biochemical and mechanical stimuli are combinatorially interpreted by cells to regulate Erk signaling dynamics.

P2367/B620

Atomic Force Microscopy Measurements of Lung Tissue.

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Matrix stiffness is critical for the proper phenotypic behavior of cells. This is also true for cancer cells as cancer cell function is modulated by matrix stiffness. Recent attention has been given to the mechanical properties of the tumor microenvironment, which includes blood vessels, immune cells, and the extracellular matrix, and how it can affect cancer cell proliferation, extravasation, migration, and other processes in the metastatic cascade. Studies have shown that a stiffer tumor microenvironment promotes cancer progression and cell migration in addition to contributing to other microscopic and macroscopic changes in the cell and tissue architecture. Several studies have attempted to quantify the tumor microenvironment stiffness and in doing so, have employed a variety of techniques and methods. However, there exists a wide range of values reported for similar tissues. Thus, accurate and precise ways to measure matrix stiffness must be developed. Here, we find that tissue preparation prior to mechanical testing significantly impacts the elastic modulus value obtained. We use atomic force microscopy to measure the elastic modulus of fresh mouse and human lung tissues. We test samples that are cryopreserved, fixed with paraformaldehyde or infiltrated with sucrose. Our data indicate that time post-mortem and fixation alter mechanical properties. We also find that sectioning the tissue before mechanical measurement as opposed to measurement on the bulk sample affects the measured stiffness values. Further, our data indicate that the method of sample preparation can alter the micromechanical properties of lung, and reproducibility of measurements relies on consistency of preparation protocols.

P2368/B621

Extracellular Matrix Stiffness Regulates Chondrocyte TRPV4 Activity.

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Osteoarthritis (OA) is the most common joint disorder in the United States, affecting over 54 million adults. During the development of OA, the stiffness of the extracellular matrix (ECM) of cartilage decreases due to a degradation of both collagen type 2 and proteoglycans. The Transient Receptor Potential Vanilloid 4 (TRPV4) channel plays a crucial role in the development, function, and mechanosensation of cartilage through its conductance of calcium. Whereas altering matrix stiffness changes the function and phenotype of chondrocytes, few studies have investigated the role ECM stiffness has on TRPV4-mediated calcium signaling and chondrocyte function. We postulated that ECM stiffness would modulate TRPV4 activity to alter chondrocyte phenotype and function. Here we show that decreasing the ECM stiffness, modeled by poly(ethylene-glycol) (PEG) hydrogels, reduces the responsiveness of TRPV4 to hypotonic challenge. PEG-diacrylate (PEG-DA) hydrogels were made using weight percentages of 14.1% (350kPa), 9.8% (175kPa), and 5.2% (35kPa) of PEG-DA were used to mimic normal, arthritic, and severely arthritic cartilage, respectively. ATDC5 chondrocytic cells seeded on the

hydrogel surface were loaded with Fluo-4 before recovering in either HBSS or 100nM GSK2193874 (GSK219), a TRPV4 specific antagonist, before challenge with 50% hypotonic swelling (HTS). The normalized intracellular calcium influx of the cells demonstrate significant differences based on the stiffness of the gels. Cells grown on the 350 kPa gels show significantly higher responses to HTS when compared to cells grown on the other gels. Additionally, cells grown on the 175 kPA gels show a significantly greater response to HTS when compared to cells grown on the 35kPA gels. Inhibition of TRPV4 with GSK219 significantly suppressed the response of cells to HTS when they were seeded only on the normal and OA gels. The balance of TRPV4 activity may play a key role in the progression of OA, as other studies indicate ablation or over-activation of TRPV4 cause deleterious effects on the phenotype of chondrocytes. Further studies will explore the role of ECM stiffness on TRPV4 and chondrocyte function and phenotype. Our findings indicate that as the stiffness of the ECM decreases, the response of chondrocytes to HTS is suppressed in a TRPV4-dependent mechanism.

P2369/B622

Substratum Stiffness Regulates TRPV44 4 Activity in Lung Epithelial Cells: Implications for Lung Development.

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Transient Receptor Potential Vanilloid 4 (TRPV4) is a mechanosensitive non-selective ion channel that is highly expressed in the lung and has been implicated in pulmonary fibrosis, hypertension, and mechanical ventilation barotrauma in premature babies. While implicated in a variety of lung pathologies, the role of TRPV4 in lung development is still unclear. Our lab has previously shown that TRPV4 is present in the embryonic lung and that it regulates airway branching and maintenance of the pulmonary vasculature during development. However, the signaling mechanism through which TRPV4 regulates these functions remain unknown. Given that cell contractility, basement membrane thinning, and correct spatial deposition of extracellular matrix (ECM) proteins are required for proper airway branching morphogenesis, we sought to understand the role of ECM stiffness in altering lung epithelial cell signaling. Whereas Rahaman et al. 2016 have shown that the response to TRPV4 agonism decreases with decreasing ECM stiffness in fibroblasts isolated from fibrotic lung tissue, TRPV4 response has yet to be studied in lung epithelial cells. We hypothesize that decreasing matrix stiffness will alter the responsiveness of lung epithelial cells to TRPV4 agonism. Here we demonstrate that that decreasing the ECM stiffness, modeled by polyacrylamide gels, reduces the responsiveness of TRPV4 to antagonism. Mouse lung epithelial (MLE) cells were seeded on top of polyacrylamide gels of different stiffnesses (2 kPa, 10 kPa, and 40 kPa) and fuctionalized with fibronectin. MLE cells were cultured for 4 days and loaded with Fluo-4 AM to assess intracellular calcium influx. MLE cells were then imaged on a Zeiss AxioObserver Z1 and challenged with 25 µM RN-1747, a TRPV4 specific agonist, and intracellular calcium was measured over a ten minute period. Our results demonstrate that as substratum stiffness decreases, MLE calcium influx decreases with TRPV4 agonism. Cells on the 40 kPa gels had the highest sensitivity to TRPV4 agonism with the largest number of cells responding and the largest transient calcium peaks. Whereas MLE cells grown on both the 2 kPa and 10 kPa responded to TRPV4 agonism, fewer cells responded and the transient calcium influxes were attenuated. These data suggest that ECM stiffness regulates the activity of TRPV4 in mouse lung epithelial cells to modulate epithelial Ca^{2+} signaling. These data, in combination with our previous work, demonstrate an important role for spatial patterning of the ECM and TRPV4 activity in regulating proper lung development.

P2370/B623

Study of Mechanical Response of Hepatic Cell Line C9 to Substrate Stiffness.

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Mechanical interactions are key mediators of cellular processes such as proliferation, differentiation, motility and quiescence. Thus motivating the use of substrate stiffness control for mechanotransduction studies showing that lower stiffness is expected to maintain epithelial phenotype and may induce a modification on mesenchymal phenotype as well. Although the stiffness values for different types of adherent cells vary widely, it is generally true that cell proliferation and differentiation increase with matrix stiffness. For most of these studies, researchers have used a wide variety of immortalized cell lines in order to guarantee experiment reproducibility from one laboratory to another. However, these cell lines were developed to survive and proliferate on commercial dishes and well plates made with very stiff materials, hence restraining their mechanical response. In particular, C9 hepatic cell line is a highly proliferative cell primed on stiff materials and a great substrates adhesion capacity when compared to other lines such as HepG2.In this work, we studied the mechanical response of C9 cells cultured on soft PDMS substrates (Young's Modulus ranging between 24 - 232 kPa) compared to a Petri dish (GPa). It was observed that by decreasing the rigidity of the substrate, it is possible to modulate the proliferation rate without decreasing cell viability. A decrease in phospho-Erk1/2 measured by Western Blot suggested that MAPK signalling pathway may be responsible for proliferation regulation rather than Hippo pathway. In spite of the epithelial morphology presented in these cells, Western blot characterization revealed that C9 presented a mesenchymal phenotype rather than epithelial. Interestingly, when maintaining the line during several passes (up to 10) on soft PDMS, C9 cells unexpectedly acquired an elongated morphology and re-established their proliferation rate as if cultured on rigid substrates. Expression levels of α-SMA in passage 10 showed by Western blot characterization suggested that cells underwent a transdifferentiation to myofibroblast. This results raise important questions regarding the use of cell lines for stiff substrates and priming on soft substrates.

P2371/B624

Effect of Substrate Stiffness and TGF-β Factor on EMT in Primary Rat Hepatocytes.

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Primary hepatocytes are epithelial cells that, when seeded on rigid substrates (like polystyrene Petri dishes or glass coverslips), their phenotype change and undergo an epithelial-mesenchymal transition (EMT)-like, which typically occurs from the third day of culture. The plastic cell culture plates and coverslips have a much greater stiffness (GPa) with respect to the reported pathophysiological values of the liver (<5 kPa). This reported transition on rigid substrates is accompanied by an increase in cortical actin expression, which has been linked to processes such as fibrogenesis; also, there is an increase in cell area spreading, in the mesenchymal phenotype markers such as vimentin and, on the other hand, a decrease in epithelial phenotype markers such as the E-cadherin. Furthermore, while in *vitro* the transforming growth factor beta (TGF-β) provokes the hepatocytes EMT[1], in *vivo* it does not have the same effect. Therefore, we decided to study the influence of this factor on primary rat hepatocytes seeded on softer substrates and assess whether there is the same epithelial-mesenchymal transition

effect. For this purpose, glass coverslides and polyacrylamide hydrogels with a stiffness of 1 kPa and 20 kPa (conjugated with collagen-I on its surface) were used. The hepatocytes were seeded on them and the cell culture was maintained for 5 days, then the cells were fixed and the expression of vimentin, E-cadherin, morphology and cellular area was determined. The results obtained suggest that TGF-β effect in the EMT is affected by the stiffness of the substrate, the rat hepatocytes on the 1 kPa hydrogels do not show their typical in vitro EMT as reported when they are seeded in tissue culture polystyrene plates with TGF-β. This project was funded by PAPIIT, UNAM #IT102017 and BDB thanks the DGAPA-UNAM Postdoc Fellowship. 1) Kaimori A., et al. Transforming Growth Factor-β1 Induces an Epithelial-to-Mesenchymal Transition State in Mouse Hepatocytes in *Vitro* (2007). The Journal of Biological Chemistry. 282:30, 22089 -22101

P2372/B625

Intracellular Molecular Cues Guide Cell Polarization on Geometrically Confined Extracellular Matrix.

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Cells recognize and properly respond to diverse biophysical stimuli to maintain mechanical homeostasis that is crucial to control cell behaviors such as cell polarization, proliferation, and migration. Biological aging is the time-dependent deterioration of cell function, resulting in the progression of chronic diseases including cancer, neurodegeneration, and cardiovascular disorders. Accordingly, the ability of cells to mechano-sense their microenvironments diminishes with biological aging. While previous studies on aging have mainly focused on the clinical aspects of tissues and organs, however, underlying mechanism of cellular aging in the context of interaction between cell and micro-environment remains unclear. Here we first identify molecular basis of attenuated cellular mechanosensation. We synthesized collagen-coated polyacrylamide hydrogels with varying stiffness to provide various ranges of mechanical strength mimicking in *vivo* tissue environments. To determine age-dependent differential mechanosensation, human dermal fibroblasts (HDFs) were obtained from differently aged healthy individuals ranging from 2 to 92 years old. For the systematic analysis of cell morphology, automated high-throughput cell phenotyping (HtCP) technique was applied. To identify the role of lamin A/C, a nuclear membrane protein mediating age-dependent functional decline of cells, young cells were lamin-mutated by transient transfection of lamin Δ50, which mimics old cells. We further quantified intranuclear dynamics by analyzing displacement of chromatin centroids with confocal microscope-based live cell imaging and high-speed particle tracking technique. Statistical analysis of mean squared displacement was performed by a customized MATLAB code. This research reveals underlying molecular mechanism of age-dependent progressive decline in single cell resolution. Using not only cellular structural markers but intranuclear protein markers and transcription factors, we estimate the grade of cellular functions when mechanical interplay occurs between cells and microenvironment. Our results reveal underlying subcellular mechanism of how age-induced malfunctioning of nucleus can attenuate mechanosensation of cellular microenvironment. We expect that the relationship between cellular age and mechanosensing ability could determine donor's biological age and health condition. The result of this research will accelerate the development of clinical applications and translational research for designing personalized medicine and recovering health condition against the aging process.

P2373/B626

Force-induced Recruitment of Cten Along Keratin Network.

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The cytoskeleton provides structural integrity to cells and serves as a key component in mechano-transduction. Tensins are thought to provide a force-bearing linkage between integrins and the actin cytoskeleton; yet, direct evidence of tensin's role in mechano-transduction is lacking. We here report that local force application to epithelial cells using a micron-sized needle leads to rapid accumulation of cten (tensin 4), but not tensin 1, along a fibrous intracellular network. Surprisingly, cten-positive fibers are not actin fibers; instead, these fibers are keratin intermediate filaments. The dissociation of cten from tension-free keratin fibers depends on the duration of cell stretch, demonstrating that the external force favors maturation of cten-keratin network interactions over time and that keratin fibers retain remarkable structural memory of its force-bearing state. These results establish the keratin network as an integral part of force-sensing elements recruiting distinct proteins like cten and suggest the existence of a novel mechano-transduction pathway.

P2374/B627

Surface Topography Modulates Actin Cytoskeleton and Cell Mechanosensing of Extracellular Matrix.

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The interaction between the cell membrane and the extracellular matrix is crucial for many cellular functions by modulating mechanosensitive signaling pathways. Physical properties of the extracellular matrix such as stiffness and topography affect such interactions. Our recent work reveals that surface topography of tens to hundreds of nanometer scale modulates cell signaling by activating intracellular curvature-sensitive proteins. We use vertical nanoscale structures protruding from a flat surface as a platform to induce high curvatures on the cell membrane. We found that these high membrane curvature induce a drastic reorganization of the actin cytoskeleton, a reduction in the number of focal adhesion, and also a reduction of the cell membrane tension. Mechanistic studies reveal that the effect arises from the activation of curvature-sensitive proteins.

P2375/B628

Studies of Polyhydroxyalkanoate Based 3d Substrates for Medical Applications.

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Biopolymers represent one of the leading sectors for bio-based products and their expected growth is foreseen to be significant within the next years. Polyhydroxyalkanoates (PHAs) represent a class of optically active biodegradable polyesters accumulated by numerous bacteria as discrete intracellular granules or as a net like extracellular structures. PHAs are non-toxic materials and biodegrade to harmless products in the environment. The work focused on developing and optimizing manufacturing process of PHA based substrates with porous structure. Additionaly the analysis of impact of PHA based substrates on mammalian cells physiology and morphology has been tested. The cytotoxicity assessment and long-term microscopic studies revealed high biocompatibility level of PHA polymer and no toxic impact on living cells. Further research concerned advanced analysis employing confocal and

fluorescent microscopy. The received data indicates the influence of biopolymer on the structure and morphology of the cytoskeleton and cell shape. The structure of actin filaments suggest a high level of cell adhesion to the biopolymeric substrate while compared with glass. A quantitative analysis was also applied to determine the impact that PHO exerts on both cells' morphology, cytoskeleton architecture and migration velocity. PHAs can be subjected to simple modifications that can change both their biological and chemical properties, as well as their physical properties such as hydrophobicity or hardness. These features make polyhydroxyalkanoates versatile polymers, which applications and commercial use can be dictated by the needs of the market and not by limitations posed by the material itself. Thanks to employment of advanced experimental methods combined with complex data analysis it was possible to precisely elucidate the impact of biopolymeric substrates on living mammalian cells and confirm their suitability in biomedical devices and medical applications.

P2376/B629

Mapping Mesenchymal Stem Cell Heterogeneity Via Single Cell Rna Profiling of Matrix-directed Differentiation Towards Soft and Stiff Tissue Lineages.

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Mesenchymal stem cells (MSCs) form a heterogeneous population of multipotent progenitor cells that contribute to tissue remodeling, repair and homeostasis. While differentiation of MSC populations towards soft and stiff tissue lineages is directed by matrix mechanics, single cells differ by their matrix-sensing potential and multilineage differentiation capacity. Here we cultured bone-marrow derived human MSCs on soft and stiff matrices that mimic fat and pre-calcified bone and used a bipotential adipogenic/osteogenic medium to induce differentiation. To study lineage specification heterogeneity, we employed the droplet-based inDrop labeling platform to perform single cell RNA (scRNA) profiling of thousands MSCS at naïve, matrix-conditioned and early differentiating states. At the population level, cell mechanosensitivity was validated by cell and nucleus projected area analyses and functional differentiation assays favoring adipogenesis on soft matrices and osteogenesis on stiff matrices. Yet, scRNA profiling indicated that matrix elasticity directs linage differentiation only in a fraction of cells. Dimensionality-reduced transcriptomes of thousands of cells collected at different time points clustered into distinctive subpopulations linking matrix priming and linage specification. Using machine learning classification tools, we identified surface marker distributions of MSC subpopulations. Diffusion pseudotime reconstruction of naïve states, matrix-priming and lineage specification histories revealed a cell-fate decision-making bifurcation towards fat and bone fates. Adipogenesis was retarded on stiff matrices and soft matrices activated chondrogenic markers in osteogenic cells. Differential gene expression screening between matrix-sensitive cells that differentiated in parallel with matrix mechanics and matrix-insensitive cells that exclusively express stress-associated surface markers revealed tropomyosin isoforms whose signaling functions we validated via knockdown and overexpression experiments. Taken together, our work provides mapping of MSC subpopulations characterized by multilineage differentiation capacity associated with cytoskeletal proteins that mediate mechanical signaling.

P2377/B630

Stiffness Regulation of Transcriptional Cofactors in Hepatocytes.

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The liver metabolizes several endogenous and exogenous molecules sustaining homeostasis in the organism. Hepatocytes are the cells that carry out all metabolic processes in the liver. It has been shown that mechanical cues affect hepatic functions because of the gene regulation and the polarity that preserves hepatic phenotype is lost. It is also known that YAP and TAZ proteins take part in regulating hepatocyte proliferation during regeneration and cancer development. However, neither YAP and TAZ nor other cotranscriptional cofactors activation by changes in stiffness has been evaluated. So, we decided to investigate the regulation of YAP, TAZ, MRTF-A and MRTF-B transcriptional cofactors by stiffness. We fabricated polyacrylamide hydrogels from 0.5 to 40 kPa to evaluate nuclear translocation of all cofactors. We also studied protein association among these mechanosensitive proteins. We found out that the main transcriptional cofactors that respond to external mechanical cues can be activated differentially and association can be regulated by stiffness in hepatocytes.

P2378/B631

Structural Relationship between the Putative Hair Cell Mechanotransduction Channel Tmc1, Tmem63/osca and Tmem16 Proteins.

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Our senses of touch, hearing, balance, and proprioception depend on mechanically gated ion channels, which transduce mechanical energy into electrical signals that are transmitted to the brain. Previous studies on the mechanisms of hearing have elucidated the biophysical properties of the mechanotransduction (MET) channel essential for hearing, yet its molecular identity and structure remain elusive. The transmembrane channel-like protein (TMC1) localizes to the site of the MET channel, interacts with the tip-link responsible for mechanical gating, and genetic alterations in TMC1 alter MET channel properties and cause deafness, supporting the hypothesis that TMC1 forms the MET channel. We generated a structural model of TMC1 based on the X-ray and cryo-EM structures of the TMEM16 and TMEM63/OSCA proteins, revealing the presence of a large cavity near the protein-lipid interface that harbors the two TMC1 mutations that cause autosomal dominant hearing loss (p.M418K and p.D572N/H), suggesting that it could function as a permeation pathway. We also find that hair cells are permeable to 3kDa dextran labeled with Texas-Red, and that dextran permeation requires TMC1/2 proteins and functional MET channels, supporting the presence of a large permeation pathway and the hypothesis that TMC1 is a pore-forming subunit of the MET channel complex.

P2379/B632

Piezo1-sAC-IP_3R2 Axis Regulates the Adaptive Cellular Responses of Endothelial Cells to Mechanical Cues.

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Mechanosensitive channels expressed by Endothelial Cells (ECs) are responsible for sensing mechanical cues and converting them into chemical signals to elicit specific cellular responses. The mechanosensitive channel Piezo1 plays an important role in vascular homeostasis by sensing hydrostatic pressure and shear stress in ECs. However, specific cellular mechanisms involved in Piezo1-mediated mechanotransduction remain elusive. Here, we investigated the mechanisms of Piezo1-mediated Ca^{2+} signals in ECs. Using intracellular and endoplasmic reticulum (ER) calcium sensors to respectively monitor the changes in Ca^{2+}_I and Ca^{2+}_{ER} concentrations, we have shown that pharmacological activation of Piezo1 induced an increase in Ca^{2+}_I that was potentiated by Ca^{2+} release from ER stores. Ca^{2+} influx through Piezo1 led to a transient activation of soluble adenylyl cyclase (sAC) which, in turn, activated cAMP-dependent Ca^{2+} release via Inositol Trisphosphate Receptor 2 (IP_3R2), a calcium channel located in the ER. Depletion of either sAC or IP_3R2 markedly reduced the Piezo1-mediated increase in cytosolic Ca^{2+}_I indicating that Ca^{2+}_{ER} release represents a mechanochemical positive feedback loop eliciting Ca^{2+} signals in ECs. Furthermore, depletion of IP_3R2 blocked alignment of ECs in the direction of shear flow suggesting that activation of IP_3R2 downstream of Piezo1 is an essential element of a mechanotransduction response. Our data, for the first time, establish the role of Piezo1-sAC- IP_3R2 axis in regulating the adaptive cellular responses of ECs to mechanical cues.

P2380/B633

Myosin-II Mediated Traction Forces Evoke Localized Piezo1-dependent Ca^{2+}Flickers.

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Piezo channels transduce mechanical stimuli into electrical and chemical signals, and in doing so, powerfully influence development, tissue homeostasis, and regeneration. While much is known about how Piezo1 responds to external forces, its response to internal, cell-generated forces remains poorly understood. Here, using measurements of endogenous Piezo1 activity and traction forces in native cellular conditions, we show that actomyosin-based cellular traction forces generate spatially-restricted Ca^{2+} flickers in the absence of externally-applied mechanical forces. Although Piezo1 channels diffuse readily in the plasma membrane and are widely distributed across the cell, their flicker activity is enriched in regions proximal to force-producing adhesions. The mechanical force that activates Piezo1 arises from Myosin II phosphorylation by Myosin Light Chain Kinase. We propose that Piezo1 Ca^{2+} flickers allow spatial segregation of mechanotransduction events, and that diffusion allows channel molecules to efficiently respond to transient, local mechanical stimuli.

P2381/B634

Myosin-dependent Mechanosensory Adaptation in *Drosophila*.

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Mechanosensory receptor cells detect and convert a diverse range of physical forces such as sound, vibration and stretch into biological (electrical) signals. The fruit fly *Drosophila melanogaster* possesses specialized organs, chordotonal organs (ChOs), to “hear” external sound, feel airflow and keep track of body motions (propiosensing). Mechanoelectrical transduction in these organs is typically controlled by active, force-generating processes (adaptation motors). The nature of those force generators, however, is not known. We have combined electrophysiological analysis with mechanical stimulation, and have correlated mechanical properties and active manipulation with neuronal activity. We show that non-muscle myosin II activity in ChOs of *Drosophila* larvae is responsible for both mechanosensoy adaptation and neuronal responsiveness. Mechanical experiments suggest that elasticity and pretension in the ChO’s depend on the activities of myosin motors.

P2382/B635

Piezo-like Channel Functions as a Mechanosensitive Regulator Governing Reproduction in *Caenorhabditis Elegans*.

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PIEZOs are mechanosensitive ion channels; the two PIEZO proteins in humans are involved in a wide range of developmental and physiological processes. Dysfunctional alleles of PIEZOs cause at least six diseases. However, the cellular and molecular mechanisms of PIEZOs in these diseases are not well understood. To address this, we investigated the functional roles of *pezo-1*, the sole PIEZO ortholog in *C. elegans*, using CRISPR/Cas9 and other genetic tools. GFP::PEZO-1 widely expresses from embryos to adults in *C. elegans*. Notably, it strongly expresses at several valvular tissues, including the pharynx-intestine and spermatheca-uterine valves. It is also expressed in the somatic gonad and in sperm. Two large deletions and multiple patient-specific loss-of-function and gain-of-function mutants cause severe reproductive deficiency, such as reduced brood size in *C. elegans*. In *vivo* observations show that newly fertilized oocytes undergo a variety of transit defects in and out of the spermatheca. Post ovulation oocytes were frequently damaged during spermathecal contraction. Due to PIEZO channels’ permeability to Ca^{2+} and the importance of calcium signaling in regulating spermatheca contractility, we are imaging the calcium indicator GCaMP in *pezo-1* mutants. Subtle variation of calcium release was observed in *pezo-1* mutants compared with wild type. To test possible genetic interactions between *pezo-1* mutants and cytosolic Ca^{2+} regulators, we performed a candidate RNAi screen to deplete calcium regulatory proteins in *pezo-1* mutants and wild type animals. Depletion of the ER calcium-release channel *itr-1* synergistically reduced brood size in *pezo-1* mutants. In contrast, depletion of the *itr-1* negative regulator, *lfe-2*, partially rescued the smaller brood size in the *pezo-1* mutant. Furthermore, sperm number significantly declined in 2-3 days adults of *pezo-1* mutants compared with wild type. Mating with male worms rescued reproductive deficiency in *pezo-1* mutants, suggesting that the lack of sperm in *pezo-1* mutants contributes to its reproductive deficiency. To test the specificity to sperm, we depleted PEZO-1 in different tissues, including somatic tissues, sperm and germline with auxin-inducible

degradation system (AID). A reduced brood size was observed in each tissue-specific degradation strain. Therefore, our study suggests that *pezo-1* may act in different reproductive tissues to promote proper ovulation and fertilization in *C. elegans*.

P2383/B636

Evaluating the Developmental Potential of Oocytes and Preimplantation Embryos Via in Situ Rheological Characterization.

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In vitro fertilization (IVF) treatments account for 1.7% of live births in the USA alone. Early identification of embryos with high implantation potential is required for avoiding clinical complications to the newborn and to the mother that are associated with multiple-embryo pregnancy and for shortening time to pregnancy. Previous studies have demonstrated a correlation between the mechanical properties of the embryos and their developmental capacity to implant in the uterus. We developed the MechanoPLATE - a multiplate-based glass device that facilitates continuous viscoelastic characterization of oocytes during in vitro maturation (IVM) and of embryos during preimplantation development under optimal culture conditions. Stress-strain relationships are obtained via controlled pressue system. Creep compliance measurements are fitted to a linear viscoelastic model. The developmental potential of oocytes is scored based on the capacity to complete second meiosis (MII) stage. Similarly, embryo quality is determined by the capacity to developmentally advance and reach blastulation stage. Using a bovine model, we demonstrate a non-invasive rheological method, which does not compromise the developmental potential of oocytes and embryos. In situ aspiration probes the integrated mechanical properties of the ooplasm and the surrounding zona pellucida. Only high quality Germinal vesicle (GV) oocytes that reach MII soften during IVM whereas poor quality arrested oocytes remain stiff. Changes in embryo mechanics offer means of discriminating between high-potential and low-potential embryos at early stages of preimplantation development. These mechanical profiles are consistent with a decrease in the reproductive potential of aged versus young cows and oocytes obtained from large antral follicles during the cold versus hot season. In situ rheological assessment of oocytes and embryos is compatible with visual evaluation of preimplantation embryo development, which offers non-invasive and accurate means of selecting the oocytes and embryos with the highest developmntal potential for transfer into the uterus. Our assisted reproductive technology has the potential of improving domestic animal breeding schemes and IVF clinical performance in human.

Chemotaxis and Directed Cell Migration 2

P2384/B637

Steering Cytoskeletal Dynamics with Electric Fields.

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Endogenous electric fields are present in many biological processes including at the edge of healing wounds. It is known that for many types of cells, including neutrophils, DC electric fields of the same order of magnitude as these endogenous fields direct cell migration to the cathode. In this presentation we determine the effect of DC electric fields on actin polymerization dynamics. We find that actin waves

are guided by DC electric fields for multiple cell types. Giant *Dictyostelium discoideum* cells (produced via electrofusion) allow us to measure electric field response of actin waves located far from the leading or trailing edge of a cell, and thus decouple cell polarity and shape from actin wave responses. We also use nanoridges, fabricated substrate nanotopographies, which alter the actin waves from the broad, two-dimensional waves seen on flat substrates to one-dimensional waves traveling along the nanoridges. We find that the one-dimensional waves on nanoridges respond faster to electric fields and respond by a 180 degree switch in wave direction to changes in field direction. Actin waves on flat substrates, on the other hand, respond to electric fields by a gradual turn in the preferred wave direction. This difference is also notable in the overall migratory behavior of neutrophil-like HL60 cells, which tend to gradually turn toward electric fields on flat surfaces, while switching front and back on nanoridges. When the nanoridges are aligned in competition with the electric field, i.e. In a perpendicular direction, we find that actin waves follow the nanoridges while, on the whole cell scale, migrating cells follow the electric field. This points to the possibility that different guidance signals may dominate cell behavior on different length scales. Together, our data reveal new insights into the mechanism behind cell migration via DC electric fields with actin waves playing a key role in cell response and control.

P2385/B638

Probing the Directional Motile Cue: Quantitative Study of Galvanotactic 3D Migration in Neutrophil-like HL-60 Cells.

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Migratory cells must constantly integrate physical and chemical cues within their environment for effective movement. Among these cues, electric fields have been found to reorient cells along the direction of potential difference (galvanotaxis), with particular relevance to development and wound healing in mammals. While this phenomenon has been studied extensively in a variety of cell types, the underlying mechanism(s) behind this directed movement remain enigmatic. Here we consider galvanotaxis in the context of ameboid immune cell migration. We study migration of the neutrophil-like HL-60 cell in *vitro* using 3D extracellular matrices in order to mimic the complex environment they may encounter within the body. We begin by demonstrating that these neutrophil-like cells indeed show directed motion under an electric potential. This response is rapid, with cells orienting along the potential gradient within seconds of the electric field being generated. Using Cas9-mediated gene-editing, we have constructed an inducible CRISPRi cell line that we then use to perturb gene function through gene knockdown. Single cell tracking data is used to infer quantitative motility characteristics including cellular persistence and we present preliminary results upon perturbation to a number of candidate genes potentially involved in galvanotaxis. We also contrast this migration modality with motile behavior under chemotaxis, as well as characterize the general motility characteristics of these cells in the absence of any directional cue. Due to their amenability to genetic manipulation, these HL-60 cells will provide an important model system to study the molecular basis underlying galvanotaxis in ameboid-type cells.

P2386/B639

Osmolarity-independent Cues Guide Rapid Cell Migration to Injury in Zebrafish Epidermis.

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During wound healing cells must respond rapidly to changes in their environment in order to mitigate tissue damage, on timescales too short to be controlled by changes in gene expression. To disentangle the effects of different environmental cues on wound-induced cell migration, we have studied wound healing in larval zebrafish: when the tailfin is lacerated with a needle, basal epidermal stem cells up to 300µm away from the wound polarize their cytoskeleton within tens of seconds and migrate toward the wound over the span of about 15 minutes. One cue that has been suggested to induce cell migration after injury is a sudden shift in osmolarity due to mixing of interstitial fluid and the external medium at the wound (Gault 2014). By changing the composition and concentration of ions in the external medium, we discovered that, although a change in osmolarity did promote cell migration, it was not necessary for actin polarization: cell migration was partially inhibited in all isosmotic media, but the actin cytoskeleton still polarized toward the wound in all media except isosmotic sodium chloride. By measuring cell volume after wound healing we confirmed that changes in cell volume were not sufficient to explain this differential cytoskeletal response in isosmotic media. This suggests that cells can sense tissue damage via non-osmotic, ion-specific mechanisms. Electric fields, arising from short-circuiting ion transport across the epidermis at the wound site, are a possible alternative signal of tissue damage, but it is unknown whether electric fields are able to induce cell polarization or migration towards a wound in *vivo* at short (<30 min) timescales. Consistent with this possibility, we found that applying an electric field in *vivo* is sufficient to stimulate actin polarization and rapid cell migration in the absence of a wound. Work is ongoing to directly measure the transepithelial potential across the epidermis and determine if this could be the source of an electric field that guides cell migration during wound healing. These results suggest that the overall physiology of the epidermis, including the transport of specific ionic species, has an important and unappreciated role in rapidly guiding cells towards damage.
Reference: Gault WJ *et al.* (2014). Osmotic surveillance mediates rapid wound closure through nucleotide release. *J Cell Biol. 207*(6): 767-782.

P2387/B640

Durotaxis: 'migration by Feel'.

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Directional cell motility is indispensable for embryonic development, immune response, wound repair, and cancer metastasis. Cells encounter a variety of cues that can serve as navigational guides, directing the cell where to migrate. While progress has been made understanding the molecular underpinnings of chemo- and haptotaxis, how a cell can sense and respond to changes in the mechanical properties of its microenvironment (durotaxis) remains elusive. Durotaxis is founded on the observation that cells seem to move by ‘feel’ from soft to stiff matrix. We sought to understand the pathways that regulate this phenomenon as little is understood on this front. To address this, we engineered photopolymerizable hydrogels that possess tunable gradients of stiffness. Using our system, we have obtained stiffness gradients that range from 0.5 to 95 kPa. Our preliminary data demonstrates that individual cells can durotax across a rigidity gradient. Our aim is to dissect the key molecular players of durotaxis with the intent of furthering the field’s understanding on how cells navigate their environment. In addition, we

hope that providing mechanistic insight on durotaxis will help bridge the role of physical cues in the development of metastasis and fibrotic diseases, to eventually pave the way for novel targeted therapeutics.

P2388/B641

Leveraging Population-Level Intercellular Variation of 2D Cell Shape for Mechanistic Insight into Cell Migration.

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Cell shape is a large-scale manifestation of the overall physical and functional state of a cell's cytoskeleton and other structural elements. Dynamic control of cell shape is essential for diverse cell types ranging from neurons to single-celled amoeba. For some well-characterized motile cell types (such as human immune cells and fish skin cells), it has been shown that control of a cell's shape is inherently linked to control of its motile behavior. Understanding the variation in a cell's shape, both through time and across a population of cells, can thus shed light on the molecular mechanisms involved in cell shape determination and motility. We have developed an approach to identify the most important axes of 2D cell shape variation in a population of thousands of fixed cells from a single imaging sample. In contrast to using simple handpicked summary metrics (such as cell area or circularity), our approach uses principal component analysis (PCA) to provide an unbiased, compact representation of cell shape, while maintaining the rich information embedded in the population's cell-to-cell variation. Furthermore, this allows quantitative, biologically-interpretable comparisons upon pharmacologic or genetic perturbation and sensitive detection of differences that would otherwise be obscured using simple metrics. This approach has enabled us to precisely quantify cell shape changes upon systematic perturbation of the actin cytoskeleton and other structural components like organelles. As a case study, we have mapped the shape space of human osteosarcoma U2OS cells (a motile cell type that forms dynamic cell shapes) and then quantified the effect of Rho kinase (ROCK) inhibitor Y-27632 on cell shape. Although individual Y-27632-treated, fixed cells do not show any obviously aberrant cell shapes relative to control cells, a population analysis reveals a striking change in their shape distribution. Upon Y-27632 treatment, U2OS cells exhibit a dose-dependent shift toward more polarized, crescent-shaped morphologies, and a wider range of left-right asymmetry. Along with live cell imaging, this population-level shape analysis of fixed U2OS cells suggests that ROCK inhibition may enable (1) higher migration speeds via enhanced front-back polarization and (2) more robust whole-cell turning via enhanced left-right asymmetry, pointing toward a role for ROCK signaling in limiting U2OS cells' intrinsic migratory behavior.

P2389/B642

Identifying Potential Protein-protein Interactions at the WRAMP Structure to Explore Regulation of Directional Cell Migration.

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In multicellular organisms, cells must move in a defined direction for proper development and immune cell function and for the pathological process of cancer cell invasion. The Wnt5a-receptor-actomyosin-polarity (WRAMP) structure is a dynamic protein network marked by the transient polarization of the transmembrane protein melanoma cell adhesion molecule (MCAM). MCAM polarization is accompanied

by enrichment of F-actin and myosin-IIB followed by immediate retraction of the cell rear. During periods when WRAMP structures are present in migrating cells, cells move with increased speed and directional persistence relative to periods when WRAMP structures are absent. This study aims to identify and test possible protein interactors and protein trafficking pathways required for WRAMP structure formation and activation of actomyosin contraction, rear cell retraction, and associated directional migration. We used proximity proteomics to identify potential members of the interaction network around MCAM at the WRAMP structures by fusing MCAM to the biotin ligase BioID or peroxidase APEX2 and purifying biotinylated proteins for identification using mass spectrometry. The results of this experiment show that BioID and APEX2 provide distinct but overlapping interactomes for MCAM. Using Gene Ontology enrichment analysis, we found that many of these proteins are involved in membrane trafficking. In addition, we discuss imaging methods used to validate the presence of these candidates at the WRAMP structure and monitor the role of different membrane compartments in WRAMP structure formation. This study corroborates a role for membrane trafficking at WRAMP structures and will help elucidate specific protein-protein interactions involved in controlling actomyosin activation and directional cell migration at the rear of the cell. More broadly, this work expands on the functional implications of adhesion molecule turnover through membrane trafficking and its impact on cell motility.

P2390/B643

Translation Contributes to Cell Migration in Confined Microenvironments.

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Cancer cells experience confinement in *vivo* during metastasis as they leave the primary tumor and migrate through the surrounding extracellular matrix, intravasate to the bloodstream, travel through capillaries as small as 3 μm in diameter, and as they extravasate into new tissues to form secondary tumors. Cancer cells in high degrees of confinement are able to employ novel migration mechanisms as actomyosin contractility is disrupted. In line with this, we have previously showed that low degrees or loss of actomyosin contractility have promoted differing subsets of RNAs to localize to peripheral regions of both cell protrusions into very narrow pores and cells confined within narrow microchannels. This is relevant as cells are able to direct subcellular RNA localization and local translation for migration purposes in two dimensional environments. Therefore, we sought to understand how global translation patterns may be altered and used for cell migration purposes in confinement. We hypothesized that cells in confinement would display altered patterns of translation due to the geometrical restrictions imposed upon the cell. We model in *vitro* confinement by seeding cells into 3 μm wide by 10 μm tall microchannels and encouraging them to migrate by establishing a chemoattractant gradient. Using pharmacological inhibitors and translation imaging methods, we explore the role of RNA translation during migration in confinement. Interestingly, our current findings point to a role for RNA translation during changes in directionality. We will discuss recent findings and our efforts to delineate the underlying mechanism.

P2391/B644

Mapping the Heterochromatin Changes Upon Induction of Cell Migration and Revealing the Role of H3k27me3 in Determining the Transcriptome of Migrating Cells.

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H3K9me3, H3K27me3 and H4K20me1 are epigenetic markers associated with chromatin condensation and transcriptional repression. Previously, we found that migration of melanoma cells is associated with a global increase in chromatin condensation that includes a 2-4-fold increase in these markers as detected by immunostaining. This heterochromatinzation is required for efficient cell migration. Our studies together with recent reports by others suggest it is a general signature of migrating cells. In this study, we mapped these markers by ChIP-seq to get deeper understanding of their function. The analysis revealed that induction of migration leads to expansion of these markers along the genome and to an increased over-lapping between them. Significantly, induction of migration led to a higher increase in H3K9me3 and H4K20me1 signals at repetitive elements than at protein-coding genes, while an opposite pattern was found with H3K27me3. Transcriptome analysis detected 182 genes altered in their RNA levels 3 hours after induction of migration. These genes are associated mainly with tumor migration signaling pathways such as TGF-β and ERK5, energy generation and oxidative stress response. Inhibition of H3K27 methylation by an Ezh2 inhibitor revealed that 33% of the migration-associated changes in RNA levels are dependent on H3K27me3. These H3K27me3-dependent genes are less prone to regulation at the RNA stability level than H3K27-indepednt migration-altered genes. Surprisingly, H3K27me3 is also required to prevent changes in the RNA levels of 501 other genes upon induction of migration. Taken together, our results suggest that heterochromatinization in migrating cells covers all genomic elements rather than restricted to specific genomic loci and that H3K27me3 is a key component in executing a migration-specific transcriptional plan as well as to buffer the genome from unwanted transcriptional changes.

P2392/B645

Rescuing DNA Damage After Constricted Migration Reveals a Mechano-regulated Threshold for Cell Cycle.

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According to the “Go-or-grow” hypothesis, cell migration and cell cycle are mutually exclusive. Transwell pores are widely used to study 3D migration and show that migration through ~2-4 μm channels suppresses cell cycle whereas migration through larger pores relieves contact inhibition and enhances cell cycle [1]. Constricted migration causes nuclear rupture and potentially DNA damage [2]. The latter remains uncertain as the results of damage markers vary. Some remain unchanged in suitably fixed cells (e.g. 53BP1) and others correlate strongly to cell cycle (e.g. BRCA1). Here, various rescuing treatments for DNA damage in constricted migration reveals a cell cycle checkpoint consistent with excess damage. We find myosin-II inhibition rescues rupture and partially rescues DNA damage in terms of the reduced foci counts of γH2AX. Co-overexpression of multiple DNA repair factors or addition of antioxidant lead to partial rescue that is independent of rupture. Cell cycle defects persist with the individual rescuing treatments, but combined treatments fully rescue both DNA damage and cell cycle suppression in constricted migration. The sigmoidal relation between cell cycle and DNA damage is consistent with DNA damage checkpoints that halt cell cycle across both early and late phases. The same sigmoidal

relation was seen for cell migration through pores of varying diameter, confirming the trend. Thus, 3D constricted migration reveals a novel 'go or grow' mechanism in which cells cease to 'grow' if they experience enough DNA damage when they 'go', and a combination of cytoskeletal force reduction and modulation of sources or repair of DNA damage is needed to rescue the damage and the growth. [1] Pfeifer, C.R., Y. Xia, ⋯ D.E. Discher. 2018. Constricted migration increases DNA damage and independently represses cell cycle. *Mol Biol Cell*: mbcE18020079. [2] Irianto, J., Y. Xia, C.R. Pfeifer, ⋯ D.E. Discher. 2017. DNA Damage Follows Repair Factor Depletion and Portends Genome Variation in Cancer Cells after Pore Migration. *Current biology : CB*. 27:210-223.

P2393/B646

The Contractile Vacuole Polarizes and Is Critical for Cell Streaming during Aggregation in *Dictyostelium Discoideum*.

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Distinct changes often occur in morphology during the formation of cellular polarity. In the case of directed migration, cells often form a defined front and rear, as they migrate towards or away from migratory cues. In many cell types, the microtubule organizing center and nucleus take up discrete localizations relative to one another. During the directed migration that takes place in cellular aggregation, the early process in fruiting body development, *Dictyostelium discoideum*cells reorganize many cellular components, including their actin and microtubule cytoskeletons. We have discovered that the contractile vacuole (CV) network also polarizes towards the rear of the cell during migration. Observations with light microscopy of retrograde CV movement were confirmed by imaging cells expressing the fluorescently tagged CV marker dajumin. CV diastole is coordinated with propagating cAMP waves during cell streaming. Interestingly, we found that the cAMP transporter ABCC8, which has been shown to be responsible for cAMP secretion, localizes to the CV network. Mutants lacking the huntingtin protein (htt-) lacked a detectable CV in the vegetative state and had smaller than averaged sized CVs. htt- cells made very weak cAMP waves and didn't stream. htt- cells have previously been shown to have an inability to regulate their osmotic pressure, and the loss of this dynamic organelle is likely critical to this phenotype. Our results demonstrate that posterior redistribution of the contractile vacuole in migrating cells plays a critical role in recruiting cells into streams and contributes to cAMP secretion/relay.

P2394/B647

Mechanical Coupling of Epithelial Cell Migration and Actomyosin Cable Formation in the Two-layered Epidermis of Embryonic Zebrafish.

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During wound healing of the embryonic epidermis, restoration of the epidermal boundary is governed primarily by two cytoskeletal processes: migration of epithelial cells toward the wound and contraction of an actomyosin cable in cells along the wound margin. Previous studies in Drosophila epithelial tissues and in cell culture demonstrated that wound repair proceeds in distinct phases and requires coordination between both processes in mono-layered systems [1]. It remains unclear exactly how these two processes are mechanically coupled or the extent of their interaction during wound healing. In zebrafish embryos, cable formation and cell migration are spatially separated in the two layers of the

epidermis: cable formation in the superficial cell layer and cell migration in the basal cell layer. This separation offers the opportunity of pinpointing the distinct mechanical contributions of each process with cell layer-specific gene expression. Cytoskeletal actin is crucial in both cable formation and cell migration. In each layer separately, we expressed a genetically encoded construct derived from gelsolin-1 (DeAct-GS1) that directly interferes with the polymerization of F-actin by sequestering actin monomers [2]. Fluorescent microscopy and image analysis reveal that cells in the basal layer expressing DeAct-GS1 have fewer protrusions, smaller lamellipodial fronts, and do not polarize towards the wound as much as control cells. They also appear to initiate independent migration more slowly than cells expressing control constructs. Mosaic expression of DeAct-GS1 is not sufficient to inhibit wound closure. In future work, we will compare the effects of our DeAct construct to the effect of a drug with known actin monomer sequestering activity, latrunculin. Additionally, we will compare stoichiometric inhibition of actin with our DeAct-GS1 to more potent catalytic constructs in both basal and superficial layers. This will enable us to investigate the effects of mosaic inhibition of actin in one process to the other. With these findings we hope to improve our understanding of wound healing and how the two distinct actin-dependent processes are coupled. 1. Abreu-Blanco, M. T. et al. J Cell Sci 125, 5984-5997 (2012). 2. Harterink, M. et al. Nature Methods 14, 479-482 (2017).

P2395/B648

Algal Mind-control: Phototaxis of the Giant Ciliate *Stentor Pyriformis* Is Mediated by Algal Endosymbionts.

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Photosynthetic algae are responsible for ~50% of global primary production of biomass. Algal endosymbiosis is recognized as a major driver of the evolutionary diversification of eukaryotes, conferring complex cell biological functions such as phototrophy and phototaxis to their hosts. As single cells, ciliates harboring photosynthetic endosymbionts are common in aquatic ecosystems, yet how single cells integrate symbiont-derived light sensing remains mysterious. Here, we describe the first positive phototactic behavior in a Stentor species, *Stentor pyriformis*, a giant single cell harbouring several hundred algal endosymbionts. Tracking single cell swimming behaviors, we have found *S. pyriformis* phototaxis behaves in a circadian rhythm as has been previously described in the green alga *Chlamydomonas reinhardtii*. Using bandpass filters across the visible light spectrum, we show *S. pyriformis* swims preferentially towards yellow-green light, supporting an algal photoreceptor-based phototactic mechanism. Additionally, minimum evolution phylogeny of the algal endosymbiont's 18S rRNA and RbcL genes identify the symbiont as a species of Chlorella most closely related to *Chlorella variabilis*, which has been shown to be sensitive to yellow-green light. Taken together, our results suggest phototaxis in the giant ciliate *S. pyriformis* is mediated by its Chlorella symbionts in a time of day and light quality manner. As an emerging model of cell biological photosymbiosis, *S. pyriformis* provides an excellent system to uncover symbiont-mediated host behavior in an organism lacking a traditional nervous system.

P2396/B649

Inter-species Repression of Diatom Light-dependent Motile Responses.

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Diatoms are unicellular siliceous golden algae, crucial to the ecological stability of numerous marine and freshwater ecosystems. Previous studies on light-stimulated direction change in diatoms indicated that *Stauroneis* (*Stauroneis phoenicenteron*) cells had increased response times when in the presence of *Craticula* (*Craticula cuspidata*) cells (*Diatom Res.*, 2016, **31**:173-184). Recent experiments have shown that *Stauroneis* cells have an even greater repression of response when they are in the presence of *Pinnularia* (*Pinnularia viridis*) cells. To determine if direct or near-direct cell-cell contact was responsible for this effect (in contrast to soluble factors in the medium), *Stauroneis* were placed in a slide chamber containing *Craticula* or *Pinnularia* on only one side of the chamber, so that some *Stauroneis* had no nearby other diatom cells, while other *Stauroneis*, immersed in the same medium, were directly surrounded by other *Craticula* or *Pinnularia*. The nearby presence of *Craticula* resulted in significantly longer direction change response times (56 ± 5 s; $P = 0.05$) compared to isolated *Stauroneis* cells in the same medium (38 ± 4 s). The nearby presence of *Pinnularia* resulted in an even greater direction change response times (139 ± 13 s; $P < 0.01$) compared to the isolated *Stauroneis* cells (68 ± 9 s). This response is dependent on living *Pinnularia* cells, and was no longer present when *Stauroneis* were in the presence of dead, ethanol-fixed and rinsed *Pinnularia* (response time of 47 ± 6 s; $P = 0.90$ compared to isolated *Stauroneis*). Interestingly, when motile *Stauroneis* were irradiated along with ethanol-fixed dead *Craticula*, they showed increased response times whether they were directly in the presence of the dead *Craticula* (77 ± 18 s) or just in nearby medium from the treated *Craticula* (105 ± 18 s). These studies suggest that *Stauroneis* response times are repressed in the presence of other species and that *Stauroneis*/*Pinnularia* effects requires direct inter-species interactions with live cells. Ethanol fixation does not remove all of the repressioneffects of *Stauroneis* interaction with *Craticula*, suggesting that some extracellular factors, such as small mucilage components, may still be present in the fixation/rinsing process, and are released into the medium. This work was supported by grants through the DePaul College of Science and Health, the DePaul University Research Council, and equipment purchased previously through NSF Grant IBN-9982897.

Cell-Cell Junctions

P2397/B651

An Investigation of the Mechanical and Functional Role of Vinculin in Conjunction with Alpha-catenin at Cell-cell Contacts.

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Mechanical forces, largely exerted or detected at epithelial cellular contacts, have been indicated necessary for normal cellular homeostasis in events such as collective cell migration, proliferation and glandular organization. Prior studies have implicated vinculin as a potent mechano-sensor, and an effective mechano-coupler of the cytoskeleton to cell-cell and cell matrix proteins. The functional consequences of vinculin as a mechano-transducer across cell-cell contacts, however, is not yet fully understood. Here, we hypothesize that vinculin in conjunction with alpha-catenin, plays a vital role in

regulating normal function and homeostasis in the epithelium. To test this, mutant MDCK II cell lines were generated using CRISPR to produce vinculin knock-out (KO), α-E-catenin KO, and vinculin α-E-catenin double KO cells. α-E-catenin KO cells were rescued with mutants unable to bind vinculin (α-E-catenin L344P) or that were constitutively bound to vinculin (α-E-catenin M319G/R326E). Using these cell lines, 2D assays were conducted that probed the role of vinculin in cellular migration and proliferation. The study concluded that both, collective migration and proliferation, were slower in α-E-catenin L344P and M319G/R326E cell lines compared to the control. Vinculin was further examined in 3D assays to analyze its role in acinar development. Compared to the control that manifested a single lumen morphology, the α-E-catenin KO cells, and the double KO cells, presented disrupted acinar lumens, thereby emphasizing the role of vinculin and α-E-catenin in acinar formation. To further investigate the force dependent role of vinculin and alpha-catenin, traction- force and FRET-force measurements were performed. Traction force showed only a modest, 25% decrease, in force in α-E-catenin KO cells, suggesting that the cell-cell adhesion is still mechanically preserved. Furthermore, measurement with an E-cadherin FRET force sensor showed no significant difference in force across E-cadherin in confluent monolayers, indicating that the adherens junction is still mechanically loaded in the absence of α-E-catenin. We speculate that the adherens junction is still able to remain connected to actin in the absence of alpha-E-catenin. Altogether, these data demonstrate that vinculin in conjunction with α-E-catenin, functions as a regulator of mechanical forces at intercellular junctions and cell-matrix contacts thereby regulating epithelial cellular homeostasis.

P2398/B652

Investigating Vinculin's Role in Responding to Locally Applied Mechanical Force at Cell-cell Junctions in Vertebrate Epithelia.

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Cell-cell junctions, including adherens junctions (AJs), which promote cell-cell adhesion, and tight junctions (TJs), which promote epithelial barrier function, are linked to an apical array of F-actin and Myosin-II (actomyosin). This linkage to the actin cytoskeleton is important for maintaining the structure and function of these cell-cell junctions. Vinculin, a scaffolding protein, is recruited to AJs when tension is increased at cell-cell junctions in order to reinforce the connection between AJs and actomyosin[1]. However, how epithelial cells maintain adhesion and barrier function when mechanical force is locally applied on cell-cell junctions – at the cleavage furrow during epithelial cell division or when three cells pull on a single point at tricellular junctions – remains unclear. We have shown that Vinculin is recruited to reinforce AJs at the cleavage furrow during cytokinesis, and expression of dominant negative Vinculin results in a reduction of AJ and TJ proteins at the cleavage furrow[2]. Vinculin also exhibits tension-dependent enrichment at tricellular junctions. Using *Xenopus laevis* embryos, we are knocking down Vinculin to investigate its role in response to both naturally occurring local increases in tension as well as manipulations that globally induce tensile stress. Preliminary experiments suggest that Vinculin acts to counterbalance the locally applied force generated by the contractile ring. Vinculin knockdown cells with one or fewer junctions intersecting with the cleavage furrow divide faster than control cells. However, when two or more intersecting junctions are present at the cleavage furrow, Vinculin knockdown cells divide in a time frame similar to that of control cells. We propose that in this case, the force provided by the intersecting junctions acts to counterbalance the force generated by the actomyosin contractile ring. We hypothesize that Vinculin stabilizes AJs when mechanical force is applied to maintain junction integrity and barrier function. These experiments will help us gain a better understanding of how

epithelial cells maintain cell-cell adhesion and barrier function when cell-scale and tissue-scale forces challenge the connections between cells. 1. Yonemura, S., et al., *alpha-Catenin as a tension transducer that induces adherens junction development.* Nat Cell Biol, 2010. **12**(6): p. 533-42. 2. Higashi, T., et al., *Maintenance of the Epithelial Barrier and Remodeling of Cell-Cell Junctions during Cytokinesis.* Curr Biol, 2016. **26**(14): p. 1829-42.

P2399/B653

Dysfunction of PLEKHA7 Promotes Cell Proliferation and Correlates with the Progression of Pancreatic Ductal Adenocarcinoma.

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Cell-cell adherens junctions are crucial to the formation and maintenance of epithelial tissues. Their disruption plays a critical role in the progression of epithelial cancers. PLEKHA7 is an integral component of adherens junctions at the apical zonula adherens and has proposed tumor suppressive functions. This study aims to determine the role of PLEKHA7 in the progression of Pancreatic Ductal Adenocarcinoma (PDAC), a lethal cancer with a five-year survival rate of 8%. Endogenous expression of PLEKHA7 was knocked down following infection of BxPC3 cells with lentiviruses expressing PLEKHA7 shRNA. Additionally, PLEKHA7 was ectopically overexpressed in the same cells using PLEKHA7-expressing retroviruses. Subsequent changes to cell growth were measured using an MTT Assay. Next, over 40 human Pancreatic Intraepithelial Neoplasia (PanIN) cores were classified by stage, stained for PLEKHA7 by immunohistochemistry, and characterized for expression phenotype. Finally, over 100 human PDAC cores were stained for PLEKHA7 by immunohistochemistry and characterized for expression phenotype. In the BxPC3 cell line, knockdown of PLEKHA7 led to significantly increased cell growth, compared to a control group. Overexpression of PLEKHA7 in the BxPC3 cell line led to significantly decreased cell growth, compared to a control group. Cell growth was also studied in the PANC-1 and Capan-2 cell lines. As the human PanIN lesions progressed from stage 1A to stage 3, PLEKHA7 was increasingly lost or mislocalized from the zonula adherens. An alysis of the carcinoma cores revealed PLEKHA7 to be lost or mislocalized in the vast majority of cases. These observations are consistent with PLEKHA7's proposed tumor suppressive function and suggest a novel role for the protein in the formation and progression of PDAC.

P2400/B654

Potential Role of TNTs in Astrocytic Gliosis.

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Astrocytic gliosis induced by direct cell-cell communication(s) has been long suspected but its mechanism(s) have not been clearly identified. We present our recent research that indicates a potential role for Tunneling Nanotubes (TNTs) in A1 phenotype astrocyte reactivity. TNT formation has been reported in several cell systems including astrocytes and neurons. A nanoscale-diameter tube originates on one cell and extends freely without substrate interactions for multiple cell body lengths, to establish a physical or secretory connection with another cell [1], as opposed to gap and adherens junctions formed between adjacent somata. TNTs appear to function as communicating junctions, and represent a mechanism that can be hijacked to communicate a diseased state at a level of cell-to-cell granularity. During our studies of astrocyte responses modulated by external nanophysical cue sets [2],

culture surface: poly-l-lysine-functionalized Aclar® (PLL Aclar) was investigated. When primary P1-3 cerebral cortical astrocytes (rat model) were stimulated with dibutyryladenosine cyclic monophosphate (dBcAMP) while modulated by PLL Aclar cues, they became reactive in ways that mimicked scar formation during gliosis, thus providing a model system for the study of A1 phenotype astrocyte reactivity. We now report that atomic force microscopy investigation of dBcAMP astrocytes modulated by PLL Aclar cues at time point 24h revealed extensive coverage of cell surfaces by TNT-like structures. Structures were typically y-shaped with cell attachments on the short branches and a long ~ 80-90 nm diameter branch that extended over one or more cell body lengths. Immunocytochemistry was performed using super-resolution microscopy, revealing long-distance cell-cell connections. The protein constituents were demonstrated to be actin, tubulin and also GFAP. GFAP has not be previously reported as a TNT constituent protein but is well known to be upregulated during astrocytic gliosis. Current investigations using Fluorescent Recovery after Photobleaching (FRAP) to investigate cell-to-cell transmission of Ca2+, up-regulated by mechanical stimulation of one cell in a connected pair, are designed to test if all or only some TNT candidate structures are capable of transmission. [1] HH Gerdes, et al., Mech. Devel. 130:381-387 (2013)[2] VM Tiryaki, et al., Nanomed. 10(4):529-545 (2015)

P2401/B655

Micropatterning of EM Grids for Cryo-electron Tomography of Endothelial Cell-cell Junctions.

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Endothelial cell-cell contacts contain adherens junctions, mediated by cadherins, that are important for controlling vascular permeability. While it is known that altered junctional organization can lead to vascular fragility, the detailed macromolecular organization at endothelial cell-cell junctions is poorly understood. Cryogenic electron tomography (cryo-ET) is increasingly being used to investigate macro-molecular protein complex structure and organization inside cells. Recently, extracellular matrix (ECM) micropatterning of electron microscopy (EM) grids via maskless photopatterning has emerged as a method to position and confine cells to specific shapes on EM grids for whole-cell cryo-ET (Engel et. Al., bioRxiv 2019). Here, we report on an EM grid micropatterning approach that improves the efficiency of our cryo-ET workflow by increasing the yield of endothelial cell-cell junctions positioned in areas accessible to the electron beam on the EM grid. We demonstrate compatibility of this EM grid micropatterning technology with vitrification and cryo-ET and aim to apply the technology to resolve the organization and structure of junctional macromolecular complexes.

P2402/B656

The Role of the Desmoplakin-Ect2 Interaction in Cardiac Function: Emerging Roles for Desmosomes as Essential Signaling Hubs.

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Desmosomes (DSMs) are intercellular junctions that anchor intermediate filaments (IF) at sites of cell adhesion in organs that withstand high mechanical forces like the skin and the heart while maintaining tissue integrity. Defects in DSM proteins cause cardio-cutaneous pathologies like Carvajal syndrome. The IF anchoring protein desmoplakin (DP) is essential for DSM integrity and is a frequent target of mutations in disease. While DP's structural role in cells is well established, new roles for DP as a signaling hub are fast emerging. Here, we identify a novel interaction between DP and the Rho Guanidine

exchange factor Ect2 and **hypothesize that DP acts as a scaffold to recruit Rho GTPases, in turn mediating changes in F-actin and cardiomyocyte (CM) contractility. To** address this hypothesis, we first assessed DPs role in recruiting Ect2 and subsequently Rho to cell borders. Using immunofluorescence microscopy in neonatal rat ventricular cardiomyocytes (NRVCMs) and tissue from a CM-specific DP-deficient mouse model (DPcKO), we found that Ect2 and active Rho (Rho-GTP) localized to cell junctions in controls while this localization is abrogated when DP was silenced in NRVCMs or absent in the DPcKO tissue. Re-expression of wild-type DPII in depleted NVRCMs restored Ect2 and active Rho border localization. However, junctional Ect2 was reduced when a DP Carvajal mutant missing the putative Ect2-binding C-terminal region (7901delG) was expressed or cardiac tissue from Carvajal patients was analyzed. These findings support the idea that **DP is required for Ect2 and active RhoA localization at cell borders in vitro and in vivo** and that this interaction is potentially impaired in Carvajal syndrome. Rho stimulation is known to induce actin stress fiber formation and promote actomyosin-mediated contractility, essential for cardiac function. Through a collagen compaction assay, we showed that DP and Ect2 silencing reduced NRVCM contractility and this was partially restored by re-expressing wild-type DPII. Impaired contractility was also confirmed through reduced levels of troponin and myosin light-chain upon DP silencing as well as disrupted F-actin structure. Taken together, our data suggest through its interaction with Ect2, DP governs Rho signaling and actin organization to ensure mechanical integrity and cardiac function. RhoA GTPase and its downstream effectors also contribute to CM identity and differentiation, raising the possibility of an expanded role for DP as an essential signaling hub in tissue morphogenesis.

P2403/B657

Irf6 Regulates Cell Adhesions Independently of RhoA.

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IRF6 regulates cell adhesions independently of RhoA an gelo antiguas, Martine Dunnwald Department of an atomy and Cell Biology Interferon regulatory factor 6 (IRF6) is a transcription factor that belongs to the IRF family of transcription factors. Mice lacking Irf6 exhibit defects of the epidermal barrier including abnormal localization of tight junction components to the upper layers of the epidermis compromising the epidermal integrity. Therefore, we hypothesize that IRF6 positively regulates the formation of cell-cell adhesions. To test our hypothesis, we took advantage of an in vitro system of keratinocyte culture. We performed cellular assays to evaluate the integrity of keratinocyte monolayers after mechanical disruptions. For this, we cultured keratinocytes until confluency, then used Dispase to remove cell-extracellular matrix adhesions, generating a floating monolayer upon which mechanical forces were applied by pipetting. Our data shows that the integrity of IRF6 deficient monolayers is disrupted compared to wild type. To understand the molecular mechanisms underlying this observation, we used immunofluorescent confocal microscopy to follow the pattern of cell-cell adhesions after inducing their formation following addition of Ca^{2+}. We found that components of the adherens junctions and tight junctions where not properly localized to the membrane, while desmosomal components Plakophilin and Desmocolin were absent from the membrane of IRF6 deficient cells. Interestingly, protein levels of junctional components were not altered in IRF6 deficient cells. Increased RhoA activity is a characteristic of cells lacking IRF6. However, the addition of a ROCK inhibitor did not change the pattern of localization of junctional proteins. To further investigate this junctional defect, we evaluated p120 catenin and found this molecule absent from the plasma membrane of IRF6 deficient cells. Overall, our data suggest a role for IRF6 in the proper localization of cell-cell junction components at the cell membrane. Our

preliminary evidence shows no recovery of the cell adhesion defect by using a ROCK inhibitor in IRF6 deficient keratinocytes, suggesting cellular processes downstream of RhoA are not the origin of this defect.

P2404/B658

Accumulation of Intercellular Stress Dictates the Expansion Patterns of the Epithelial Monolayer.

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Dynamic expansion of cellular monolayers occurs during physiological events such as embryogenesis, wound healing, and cancer invasion. The expanding epithelial monolayers often display a tortuous landscape characterized by the collective protrusion, called 'fingering,' or the individual cell scattering, called 'dissemination' at the interface of the advancing front. Until now, the physical mechanism for such diverse expansion patterns remains largely unknown. Here, we established a simple circular monolayer of MCF10A to study the epithelial expansion. Using traction force microscopy (TFM) and monolayer stress microscopy (MSM), we quantified the spatiotemporal distribution of physical forces between the cell and the substrate or between adjacent cells within the expanding monolayer, respectively. During the expansion, we witnessed the accumulation of average intercellular stress near the advancing edge. When the intercellular stresses were decomposed to radial and circumferential components with respect to the center of the monolayer, we found a strong negative correlation between the geometrical tortuosity of the expanding front and the maintenance of local circumferential intercellular stress. The radial intercellular stress component was highly accumulated at the local fingering sites, whereas the stress accumulation was disturbed at the local disseminating sites. Through the downregulation of specific cadherins (E- and P-cadherin) within the epithelial monolayer by siRNA inhibition, we revealed that P-cadherin disruption inhibited the long-range accumulation of both circumferential and radial stress components which resulted in the dissemination of majority of cells at the expanding edge. These data suggest the mechanical role of P-cadherin in physical stress transmission and accumulation within the collective monolayer. Our results emphasize the distinctive contribution of intercellular stress components to the dynamic expansion patterns of the collective monolayer.

P2405/B659

Does the Paracellular Permeability Pathway for Large Solutes, the Leak Pathway, Have a Size Limit?

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The tight junction is the primary barrier limiting the movement of solutes and water between adjacent epithelial cells (paracellular permeability). Movement of solutes through the paracellular route has been divided into two pathways. The Pore Pathway has a high capacity, mediates the permeation of small solutes (molecular radius $\leq$ ~4 an gstroms), and exhibits charge selectivity. In contrast, the Leak Pathway has a low capacity, mediates the permeation of larger solutes, and does not exhibit charge selectivity. Multiple studies have demonstrated regulation of paracellular permeability via the Leak Pathway but many questions remain about its properties and characteristics. One property of the Leak Pathway that is still unclear is whether it has a size limit for permeating solutes, i.e., is mediated by a "pore" of a

defined size, or is simply a discontinuity of the tight junction that allows the paracellular movement of solutes regardless of their size. To investigate this question, we measured the transepithelial permeation of fluorescein-dextrans of increasing size (4 kDa (Stokes radius = ~13 an gstroms) to 70 kDa (Stokes radius = ~60 an gstroms)) across post-confluent populations of the renal epithelial cell line, MDCK. Since fluorescein-dextrans cannot cross the cell membrane, the paracellular route is their primary, if not sole, pathway to move across the epithelium. Each of the fluorescein-dextrans exhibited a linear rate of transepithelial permeation. As expected, permeation rate decreased as Stokes radius increased. The Stokes-Einstein equation calculates the relationship between free diffusion rate and Stokes radius. The measured flux rates deviate from the expected relationship based on the Stokes-Einstein equation as Stokes radius increases. This argues for the existence of a Leak Pathway that is size-limited, i.e., is a “pore” of defined size. To pursue this hypothesis, we used the measured fluorescein-dextran flux rates to calculate a pore size with the Renkin sieving equation. Using the approach of Adson et al. (J Pharmaceut Sci 83:1529, 1994) we obtained a ‘best-fit” pore size of 362 an gstroms (36.2 nm) for the Leak Pathway. These results support the hypothesis that, at least under our experimental conditions, the Leak Pathway of MDCK cells exhibits a size limit which restricts the paracellular movement of solute molecules as Stokes radius increases. Combined with the ability to regulate the Leak Pathway by various physiological and pathophysiological mechanisms, these results suggest the Leak Pathway may be an important cell physiological mechanism regulating the paracellular movement of large solutes.

P2406/B660

Dynamic Endothelial Actin Structures Induce Asymmetrical Junctions That Guide Leukocyte Extravasation.

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During an inflammatory response, the activated endothelium, lining the inner layer of blood vessels attracts neutrophils that subsequently cross the vessel wall to resolve, the inflammation, a process referred to as transendothelial migration (TEM). We found that the endothelium actively recruits and guides leukocytes in their exit. Upon TNF-induced inflammation, the endothelial membrane forms dynamic dorsal and lateral protrusions. Typically, we found that actin-mediated junctional protrusions serve as preferred exit sites for neutrophils. Based on a Rac1 FRET-based biosensor, we showed that these junctional protrusions are positive for Rac1 activity and that neutrophils prefer such areas for diapedesis. In an attempt to promote the extravasation of leukocytes by locally induce Rac1 activity, we used the photoactivatable Rac1 probe. Exogenous Rac1 activation leads to local and increased TEM while blocking Rac1 activity perturbed TEM. We hypothesized that for leukocytes to successfully cross cell-cell junctions, such junctions should display asymmetric activity of membrane protrusions. To test this, we transfected one endothelial cell population with the photoactivatable Rac1 probe or constitutively active Rac mutants and cultured them with a control population of endothelial cells. This resulted in an endothelial monolayer that showed asymmetric junctions, where one endothelial cell expressed the probe and the neighboring one not. We found that almost 90% of all neutrophils had a preference to cross through the asymmetric junction by going underneath the protruding cell. These experiments show that endothelial cells generate dynamic actin structures at asymmetrical junctions to support diapedesis and not simply open cell-cell junctions as was suggested before. Our findings highlight the importance of the endothelium in recruitment and guidance of immune cells through the

vessel wall and will have an impact on therapies that are devoted to controlling leukocyte extravasation by targeting the cell-cell junction regions.

P2407/B661

Loss of ASPP2 Promotes Cell Invasion and Migration Via LSR and YAP in Human Endometrial Cancer.

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Apoptosis-stimulating p53 protein 2 (ASPP2) is an apoptosis inducer that acts via binding with p53 and epithelial polarity molecule PAR3. Although downregulation of ASPP2 is observed in endometrial cancer tissues and contributes to migration and invasion of the cancer cells in vitro, the detailed mechanisms remain unclear. Lipolysis-stimulated lipoprotein receptor (LSR) is a unique and an important molecule at tricellular contacts of normal and cancer cells, and loss of LSR promotes cell migration and invasion via upregulation of TEAD1/AREG dependent on Yes-associated protein (YAP) in human endometrial cancer cells. In the present study, to find how loss of ASPP2 promotes cell migration and invasion in human endometrial cancers, we investigated the mechanisms including the relationship with LSR and the regulation using human endometrial cancer cell line Sawano. Loss of ASPP2 by the siRNA promoted cell migration and invasion in Sawano cells. The loss of ASPP2 decrease LSR expression, and induced expression of phosphorylated YAP (pYAP), claudin-1, -4 and -7 as well as those by loss of LSR. Knockdown of YAP by the siRNA prevented upregulation of pYAP, cell migration and invasion induced by loss of ASPP2. Treatment with the specific antibody against ASPP2 downregulated ASPP2 and LSR and affected F-actin at tricellular contacts and upregulated expression of pYAP and claudin-1. The ASPP2 antibody induced cell migration and invasion via YAP. Upregulation of ASPP2 in Sawano cells was observed in high glucose condition and by treatment with HDAC inhibitors. In normal human endometrial epithelial cells, ASPP2 was in part colocalized with LSR at tricellular contacts and knockdown of ASPP2 or LSR by the siRNAs induced expression of claudin-1 and claudin-4. Loss of ASPP2 promoted cell invasion and migration via LSR and YAP in human endometrial cancer cells. The maintenance of ASPP2 plays crucial roles in preventing malignancy of cancer cells.

P2408/B662

The Rho-GEF Trio Reinforces Vascular Endothelial Barrier Integrity.

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Endothelial cell-cell junctions maintain a restrictive barrier that is tightly regulated to allow dynamic responses to permeability-inducing factors, inflammatory agents and transmigrating leukocytes. The

ability of these stimuli to remodel endothelial adherens junctions (AJs) and tight junctions (TJs) depends on Rho- and Rap-GTPase-controlled cytoskeletal rearrangements. How activity of Rho and Rap-GTPases is spatio-temporally controlled at endothelial junctions by guanine-nucleotide exchange factors (GEFs) is incompletely understood. Here, we identify a crucial role for the Rho-GEF Trio in stabilizing VE-cadherin-based junctions through indirect activation of Rap1. We show that Trio physically interacts with VE-cadherin and locally activates Rac1 at junctions during nascent AJ formation, as was judged by FRET-based biosensor techniques, resulting in the remodeling of junctional actin from radial to cortical actin bundles, a critical step for junction stabilization and TJ formation. Moreover, our data show that the GEF1 domain of Trio activates the GTPase Rap1 in a Rac1/RhoG-dependent manner. Trio-Rap1 signaling promotes the formation of linear junctions and functionally increases endothelial barrier function, in addition to thick F-actin fibers that, based on super-resolution microscopy, co-localize with active myosin II. We show that myosin-based tensile forces on F-actin bundles tightly pack endothelial cell junctions into linear arrays. By promoting an optimal junctional surface-tension ratio, Trio improves the stability of the vascular endothelial barrier.

P2409/B663

Septinpolymer Interactions with Catenin Complex Determine Epithelial Cell Polaritysite Seletion.

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Cell polarity is essential for physiological processes in metazoans which depend on the asymmetric organization of cellular components and structures. However, the molecular mechanisms underlying epithelial cell polarity establishment had remained elusive. In particular, how polarity site determination and polarity establishment machinery assembly are poorly illustrated. Here we show that septin complex interacts with catenin machinery to organize a functional domain to divide apical from basal membranes in epithelial cells using 2D and 3D model systems. Using polarized 2D epithelial cell monolayer culture model system and trans-epithelial electrical resistance as functional readout, our siRNA screen revealed the importance of septin integrity in epithelial cell polarity establishment. Our proteomic screen uncovered the dynamic formation of a novel septin-catenin complex during 2D monolayer polarization. The functional relevance of septin-catenin complex was then examined 3D culture in which suppression of septin resulted in formation of immature cyst without lumen, a hallmark seen in catenin-deficient 3D cultures and animals. Mechanistically, septins stabilize an association adherent catenin complex with actin cytoskeleton as, depletion or disruption of septin complex liberates adherent junction and polarity complexes into the cytoplasm. Using spectral imaging analyses coupled with chemical inhibitors, we are currently delineating the order of septin-catein complex assembly and polarity establishment in epithelial cells. Together, these findings reveal a previously unrecognized role for septins in the polarization of the apical-basal axis and lumen formation in polarized intestinal epithelial cells.

P2410/B664

A Novel Desmosome-COP9 Signalosome Interaction.

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The epidermis, the outermost layer of skin, serves as a critical barrier against the outside environment. A common feature among epithelial tissues is the proper maintenance and formation of cell junctional protein complexes. Desmosomes are a type of cell-cell junctional protein complex that have been shown to interact with the COP9 signalosome (Constitutively Photomorphogenic). The desmosome-COP9 complex has been shown to downregulated epidermal growth factor receptor (EGFR), which is a known signaling player in cell growth. This subsequently causes the promotion of epidermal differentiation. The mechanism by which the desmosome-COP9 complex regulate EGFR is through the removal of a protein modification called Nedd8 (de-neddylation). Upon the removal of Nedd8, EGFR is destabilized, allowing for a dampening of the growth signaling transduction pathway. While the desmosome-COP9 signalosome super-complex functions have been well defined for the maintenance of skin homeostasis, its potential role in other cytosolic regulatory pathways has not yet been explored. Previous work has lead us to question the extent to which the desmosome-COP9 signalosome can function in additional cytosolic regulator pathways. Through targeted protein analysis in a variety of epithelial cells lines, this research aims to identify and clarify the process by which the desmosome-COP9 Signalosome complex contributes to novel cell signaling.

P2411/B665

Novel Insights into Tunneling Nanotube Formation and Function.

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Abstract Tunneling nanotubes (TNTs) help in intercellular communication over long distances in animal cells and allow for bi-directional transport of cellular cargo between cells. TNTs are functionally important in several physiological processes such as signal transduction, embryonic development, immune responses, apoptosis, cancer, neurodegenerative disease initiation and intercellular pathogen transfer. Despite their established implications in health and disease, there is only a rudimentary mechanistic understanding of the biogenesis and function of tunneling nanotubes. Recently, the protein MSec was reported to be essential for TNT formation. We had hypothesized that MSec interacts with key interaction partners and needs to localize at specific subcellular regions to mediate TNT biogenesis. We describe the morphometrics of TNTs with an emphasis on the subcellular localization of MSec in mammalian cells. We observed the MSec is localized at the cortex of cells along the actin meshwork and inside the nanotubes as discrete and periodic punctae. An alysis of the MSec interactome determined in our group revealed the presence of RNA binding proteins (RBPs) that we show are required for TNT formation. Depletion of the novel MSec interacting RBPs from cells does not appear to affect the cortical enrichment of MSec, but drastically reduces TNT numbers. The strong interaction of MSec with these RBPs suggests that the localized transport to and/or translation of some of their target mRNAs at the cortex and inside TNTs could play important roles in TNT formation, maintenance and function. We further ascertained that there is significant reduction in cortical protein translation levels upon depletion of MSec-interacting RBPs. We have also uncovered a novel role for intracellular molecular motors in ensuring MSec localization to the cortex, thereby enabling TNT biogenesis. We observe a significant reduction in MSec cortical enrichment and also in nanotube numbers upon potent inhibition

of the activity of these motors using small molecule inhibitors. Present efforts are focused on understanding how molecular motors and selected RNA binding proteins regulate the process of TNT formation. Our work highlights novel molecular mechanisms and pathways that are required for tunneling nanotube formation and function and would help to explain the importance these enigmatic structures.

P2412/B666

The Crk Tyr221 Phosphorylation Site by Abl Kinase Is Dispensable for Cell Adhesion.

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ABSTRACT: the Crk adaptor proteins play a central role in many cellular processes including cell adhesion, motility and survival. There are two ubiquitously expressed Crk family proteins, Crk and CrkL, which are highly similar in their one SH2 domain and two SH3 domains. Previous studies have shown that Abl phosphorylation of the Tyr221 residue in Crk leads to an intramolecular interaction with the SH2 domain, preventing Crk from binding to other phosphotyrosine proteins. However, this elegant mechanism of Crk regulation by Abl has not been tested in vivo. Here we show that the homozygous Crk^{Y221F} knock-in mouse mutants are viable and fertile with no obvious changes in expression of Crk and CrkL protein. For integrin-mediated cell-matrix interaction, the mutant MEF cells bound normally to fibronectin-coated surface, forming focal adhesion and stress fibers after PDGF activation like wild type controls. Co-immunopreciptation experiment showed that the Y221F mutation did not perturb binding of Crk to phospho-p130Cas, which recruits Crk to the focal adhesion complex. For cadherin-mediated cell-cell interaction, the Y221F mutation also failed to perturb adherens junctions, which were readily disrupted by Abl inhibitors. Together, these results demonstrated that the Y221 phosphorylation site on Crk is not essential for cell adhesion.

P2413/B667

Interaction of the Extracellular Matrix with the Cell-cell Junction Associated RNAi Machinery in Colon Cancer.

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Colon cancer is the third most common and second deadliest type of cancer in the United States. Loss of epithelial tissue integrity is widely observed in colon tumors. Cell-cell junctions are essential for the maintenance of epithelial tissue integrity. The Adherens Junction (AJ) is a cell-cell adhesion complex composed of E-cadherin and the catenin family of proteins. We have shown that the E-cadherin-p120 catenin partner called PLEKHA7 is critical for epithelial integrity. Importantly, we have also shown that PLEKHA7 recruits the core components of the RNAi machinery, such as AGO2, DGCR8 and DROSHA at mature apical adherens junctions to regulate miRNA levels and activity. Loss of PLEKHA7 disrupts the function of the associated RNAi machinery and promotes pro-tumorigenic cell behavior. We hypothesize that PLEKHA7 acts as a sensor of epithelial homeostasis by regulating a junction-associated RNAi machinery. Prolonged wound and fibrosis are key precursors to colon cancer by extensive deposition of extracellular matrix (ECM). Investigation of 2D Caco2 colon epithelial cells grown on different ECM substrates, such as collagen, laminin and fibronectin, showed differences in the localization of PLEKHA7 and the junction-associated RNAi machinery. In addition, investigation of biomechanical effects using stiffness assays and 2D stretch assays resulted in disturbances in the proposed localization model of the

RNAi machinery at high stiffness conditions, which are a feature of fibrotic and tumor tissues. Interestingly, PLEKHA7 depletion also resulted in increased levels of a series of ECM-related proteins, revealing an extensive, bi-directional cross-talk between PLEKHA7 and the ECM. Examination of normal human colon tissues confirmed co-localization of PLEKHA7 and of the core RNAi components at the apical surface of fully differentiated colon crypts. However, this localization is disrupted or lost in colon tumor samples from patients. Together, our data point towards a novel putative tumor suppressor mechanism tethering tissue mechanosensing with cell behavior, of which we are currently investigating its modes of regulation.

P2414/B668

Influence of the Endogenous Cardiotonic Steroids Marinobufagenin and Digoxin on the Physiology of Ephitelial Cells.

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Cardiac glycosides are a group of compounds widely known for their action in cardiac tissue. In the past two decades renewed interest has emerged since some cardiac glycosides were found to be endogenously produced. Endogenous cardiotonic glycosides (ECG) modulate important features of cells. We have previously studied the effect of ouabain, an endogenous cardiac glycoside, on the physiology of Madin Darby Canine Kidney cells (MDCK), and we have shown that in concentrations in the nanomolar range, it modulates key properties of these cells, such as tight junction, apical basolateral polarization, gap junctional intercellular communication (GJIC) and Adherent Junctions. In this work we study the influence of digoxin and marinobufagenin, two other endogenous cardiac glycosides, on GJIC as well as Tight Junctions. We evaluated GJIC by dye transfer assays and tight junction sealing by trans-epithelial electrical resistance (TER) measurements, as well as immunohistochemistry and Western blot assays of expression of claudin 4. We found that both, digoxin and marinobufagenin, increase GJIC, and significantly enhance the sealing degree of the Tight junctions, reflected by increased TER. Immuno-fluorescence assays show that both compounds promote enhanced basolateral localization of claudin-4 but not claudin-2, while densitometric analysis of Western blot assays indicate a significantly increased expression of claudin-4. These changes, induced by digoxin and marinobufagenin on GJIC and TER, were not observed on ouabain resistant MDCK cells, a modified MDCK cell line that has an alfa subunit with reduced ouabain affinity, indicating that Na^+-K^+-ATPase acts as the receptor mediating the actions of both ECG. Plus, the fact that the effect of both cardiac glycosides were suppressed by incubation with PP2, an inhibitor of c-Src kinase, PD98059, an inhibitor of mitogen extracellular kinase-1 and Y-27632, a selective inhibitor of ROCK, a Rho-associated protein kinase, indicate altogether, that the signaling pathways involved include c-Src and ERK1/2 as well as Rho-ROCK. These results show that the studied ECG modulate Tight and Gap Junctions and strengthen our general hypothesis that, a very important physiological role of ECG, is the control of the epithelial phenotype and the regulation of cell-cell contacts.

P2415/B669

Dissecting the Role of the Coxsackievirus and Adenovirus Receptor (CAR) in Intercellular Communication, Hypoxia Response and Cardiac Function.

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The coxsackievirus and adenovirus receptor (CAR) is a cell-contact protein that mediates virus uptake and is essential for early cardiac development. It localizes to the tight junctions of epithelial cells and the intercalated disc of cardiomyocytes. CAR expression is low in the adult myocardium but is increased during remodeling following disease or injury. Recently, we have documented an unexpected protective role of CAR in myocardial infarction: in CAR KO mice undergoing coronary artery ligation, infarct size is reduced resulting in improved survival rates. In order to dissect weather the protective role of CAR is connected to its function in mediating intercellular communication or rather to its involvement in hypoxia signaling or cytoskeletal and extracellular matrix remodeling, we have started to generate a comprehensive interaction map and probe the protein environment depending on external stimuli. To this end we use human iPSC derived cardiomyocytes in a proximity proteomics approach with BioID-CAR fusion protein and evaluate the role of CAR in the response to hypoxia and cell death. Thus, we aim to reveal how car integrates endocytosis, cell-contact formation, and signal transduction to provide the mechanistic basis for future CAR directed therapies.

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Focal Adhesions and Invadosomes

P2416/B670

Septin 9 Is Required for Tks5-dependent Formation of Invadopodia.

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Metastatic cancer cells invade through tissue barriers by forming specialized actin-rich protrusions termed invadopodia. Invadopodia protrude into the extracellular matrix and degrade it by secreting matrix metalloproteinases (MMPs). Formation of invadopodia involves the recruitment and clustering of the scaffold protein TKS5 (Tyr kinase substrate with five SH3 domains) to PI(3,4)P2 membrane domains. Phosphorylation of TKS5 by the Src tyrosine kinase triggers the recruitment of the actin-binding protein Nck1 and a step-wise maturation of invadopodia, which is characterized by the recruitment and phosphorylation of cortactin and secretion of MMPs. A growing number of studies show that over-expression of septin 9, a member of the septin family of GTPases, enhances the migratory and invasive properties of cancer cells. However, the role of septin 9 in invadopodia formation is poorly understood. Here, we show that SEPT9_i1 functions in the early stages of invadopodia formation by affecting membrane recruitment and/or clustering of TKS5. Using SEPT9 isoform-specific shRNAs and gelatin degradation assays, we show that SEPT9_i1 depletion decreases the ability of MDA-MB-231 cells to degrade ECM without impacting overall cell area. Strikingly, SEPT9_i1 depletion results in diminished TKS5 clusters on the ventral membranes of MDA-MB-231 cells. In addition, pTyr421-cortactin puncta are also reduced indicating a loss in invadopodia precursors, which are enriched with phosphorylated cortactin. Consistent with a role of SEPT9_i1 in early stages of invadopodia formation, SEPT9_i1 appears to localize at the base of invadopodia surrounding areas of matrix degradation. On-going work aims at

determining the spatio-temporal role and mechanistic function of SEPT9 in the TKS5 pathway of invadopodia formation.

P2417/B671

Real-time Imaging of Intrinsic and Extrinsic Control Mechanisms in Invasive Breast Carcinoma Cells.

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Tumor cell structures that have long been hypothesized as necessary for metastasis are invadopodia, invasive protrusions rich in structural proteins (Tks5, actin), adhesion proteins (eg. Integrin β1), and metalloproteases. Using our unique intravital imaging approaches, we previously demonstrated that invadopodia in vivo are necessary for intravasation and consequent lung metastasis. In primary tumors, we found that cells which assemble invadopodia migrate at slow speeds, in perivascular niches where the ECM is cross-linked. Outside of these niches, no invadopodia were observed and cells migrated at high speeds, via contact guidance along collagen fibers. The invadopodia-driven motility can be switched to contact guidance by reducing the ECM cross-linking or by knocking down Tks5, which in turn reduces intravasation and metastasis. We next deduced that invadopodia-driven motility consists of two oscillating states: i. Invadopodia state, in which a cell is relatively sessile while it assembles invadopodia and degrades ECM; ii. Locomotion state. State balance is regulated by integrin β1 activation levels. Importantly, the Invadopodia state only occurs in early G1, whereas the Locomotion state can be seen throughout the entire cell cycle, suggesting that the cell cycle controls invadopodia assembly. Using FUCCI markers, we next show that Invadopodia state occurs during the G1 phase of the cell cycle. A close look at the regulators of G1 revealed that the cell cycle regulator p27kip1 localizes to the sites of invadopodia assembly and overexpression of p27kip1, but not p21cip1, causes faster turnover of invadopodia and increased ECM degradation. Taken together, these findings suggest that invadopodia function is extrinsically controlled by ECM via integrin β1, as well as intrinsically, via specific cell cycle regulators.

P2418/B672

A Synthetic Dysmobility Screen Identified STK40 to Bridge MAPK Signaling and Adhesion-based Cytoskeleton in Kinase-independent Manners.

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Cell migration is important in biological processes but how theses cell migration-related genes crosstalk with each other remain unclear. To resolve this mystery, we conducted a “two-hit” cell migration screen using short hairpin RNA (shRNA) and small molecules inhibitors. Among our candidates, Serine-Threonine Kinase 40 (STK40), a putative serine/threonine kinase, showed potential interaction with MAPK in that suppression of both genes synergistically reduced cell motility. Here we report that STK40 affects migration by altering focal adhesion (FA) dynamics. STK40 knockdown enhanced FA intensity, FA

turnover, increased stress fibers and phospho-myosin light chain (p-MLC). These phenomenon could be rescued by STK40 overexpression even when its kinase domain was truncated, indicating that STK40 might not affect FA dynamics via kinase activity. Our further elucidation of its molecular mechanism revealed that inhibition of MAPK signaling abolished the effect of STK40 on FA dynamics, symbolizing that STK40 cooperated with MAPK signaling in the same pathway. Moreover, literatures and our results showed that both MAPK signaling and STK40 promoted YAP activity, and that YAP changed FA dynamics similar to the effect of STK40. Altogether, our studies demonstrate that non-kinase action of STK40 works synergistically with MAPK signaling, activating YAP pathway to regulate FA dynamics and cell migration.

P2419/B673

Abl-mediated PI3K Activation Regulates Macrophage Podosome Assembly.

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Podosomes play critical roles in macrophage adhesion, migration, and matrix degradation. Dot-like F-actin assembly at the plasma membrane is one of the key events of podosome formation. Nevertheless, membrane signals to trigger actin polymerization at the macrophage podosome remain poorly understood. Here, we find that phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3) lipids are enriched at the plasma membrane of macrophage podosome and serve as the membrane signal to promote WASP and Arp2/3 mediated actin polymerization. We find that phosphoinositide 3-kinase (PI3K) catalytic subunit PIK3CB is distinctly enriched at the podosome core, while PIK3CA, PIK3CD and PIK3CG are not. Knockdown and chemical inhibition of PIK3CB suppress podosome formation. PI3K regulatory subunit p85alpha is recruited to podosome in a Src and c-Abl dependent manner. Inhibitions of c-Abl kinase impede PI3K activation, podosome assembly, and gelatin degradation. Src kinase inhibitions block c-Abl mediated PI3K activation. Transwell migrations of macrophages are suppressed when Src, c-Abl, or PIK3CB is inhibited.Thus, spatiotemporal biogenesis of PI(3,4,5)P3 lipids mediated by Src/c-Abl/PI3K signaling orchestrates podosome assembly, matrix degradation, and chemotactic migration of macrophages.

P2420/B674

Coupling between Actin Retrograde Flow and Focal Adhesion Molecules Visualized by Single-molecule Speckle Microscopy.

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Cell migration is important for many biological processes including development, wound healing and tumor metastasis. At the cell leading edge, the retrograde actin flow, continuous centripetal movement of the actin network, is widely observed in migrating cells. During cell migration, the retrograde actin flow has been thought to promote protrusion via linkage between the lamellipodial actin network and focal adhesions as postulated in the clutch model (Mitchison and Kirschner, Neuron, 1988). However, the role of the interaction between the retrograde actin flow and focal adhesions largely remains unclear. Single-molecule imaging is a powerful approach to directly monitor the mechanics linking actin dynamics and cell adhesion at the molecular level. In this study, we examined molecular motions of focal adhesion components and actin in lamellipodia by using Single-Molecule Speckle (SiMS)

microscopy. Interestingly, several focal adhesion components exhibit flow-associated motion along the retrograde actin flow. Furthermore, in the cell peripheral region between the cell edge and the frontal edge of focal adhesions, the retrograde flow biases toward focal adhesion. Our observations suggest that the retrograde flow streaming into focal adhesions effectively recruit focal adhesion components to focal adhesions. Our findings provide new insights into the physiological roles of the actin retrograde flow in cell migration.

P2421/B675

Mechanisms of Focal Adhesion-based Cancer Cell Migration in *Vivo*.

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Cell migration is critical during many biological processes, including embryonic development, immune surveillance, wound healing, and cancer. Cancer cell migration mechanisms in cell culture models have been well characterized. Focal adhesion complexes have been recognized as an important structure that physically link the cell to the extracellular matrix. The dynamic assembly of focal adhesion at the cell front and disassembly at cell rear are tightly coupled with actomyosin contraction force to facilitate cell migration. Cell culture studies have provided a wealth of information regarding focal adhesion biology, however, cell-matrix interactions through focal adhesion complexes during cancer cell migration in living organisms has yet to be clearly established. To address this challenge, we have taken advantage of the optically transparent, genetically tractable zebrafish larvae to manipulate both the environment and our cells of interest, the highly migratory zebrafish melanoma (ZMEL) cells. We expressed fluorescently-tagged proteins to visualize specific components of focal adhesions during cancer cell migration in *vivo*. Strikingly, we discovered that transplanted melanoma cells disseminate to the skin and form punctate structures at the melanoma cell-matrix interface. These punctate structures localize ena/VASP, and a core focal adhesion protein, Paxillin. Thus, we hypothesize that melanoma cells form focal adhesions when they attach to the skin, and this interaction is a critical step during cell migration in *vivo*. We are currently characterizing focal adhesions in *vivo* by analyzing the focal adhesion ultra-structure and quantifying their dynamics in migrating cells. We are also assessing whether melanoma cells transduce a force to the environmental extracellular matrix. These studies will provide significant insight into focal adhesion-based cell migration in *vivo*. It will also help to identify whether melanoma cells utilize focal adhesion-based motility for dissemination and thus we can gain a better understanding on how this poorly understood process contributes to cancer-related deaths.

P2422/B676

Role of Rhoa Binding Protein Slk in Muscle Hypertrophy.

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Small GTPase RhoA is involved in muscle cell proliferation and differentiation via effector proteins. We identified STE20-like kinase (SLK) as a RhoA binding protein by biotin enzyme labeling (BioID) method. Since SLK is an essential protein for embryonic muscle development, we analyzed the role of the interaction between RhoA and SLK in muscle hypertrophy model. After differentiation, RhoA and SLK protein expression increased in C2C12 myotubes. IGF-1 stimulation of differentiated C2C12 myotubes induced RhoA activation and phosphorylated SLK Ser189 residue, which is dependent on SLK kinase activity. SLK protein has a kinase domain (KD) on the N-terminal side and two coiled-coil domain (CC) on

the C-terminal side. In order to determine the RhoA binding region of SLK, recombinant proteins of GST fusion KD, CC-1, and CC-2 were verified for binding to RhoA by pull-down assay. As a result, binding to RhoA was detected in the CC-2 domain. Besides, recombinant RhoA protein substituted with $GTP_{\gamma}S$ specifically bound to SLK. In the overexpression of constitutively active mutant RhoA (G14V), RhoA (G14V) bound to SLK, and phosphorylated SLK Ser189. On the other hand, constitutively inactive mutant RhoA (T19N) inhibited SLK phosphorylation dependent on IGF-1 stimulation. Pretreatment with the SLK inhibitor erlotinib suppressed IGF-1-induced myocyte fusion and reduced muscle hypertrophy in C2C12 cell. These results suggest that SLK acts as a RhoA effector protein in promoting muscle cells differentiation in IGF-1 muscle hypertrophy.

Bioengineering of Cell-Matrix Interactions

P2423/B677

Collective Mechanosensing of Matrix Rigidity Controls Agonist-induced Calcium Oscillations and Force in Multicellular Ensembles of Smooth Muscle Cells.

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The **objective** of this study was to understand specific mechanisms by which pathological alterations in the extracellular matrix (ECM) of airways & blood vessels could be causative of diseases like asthma & hypertension. The binding of a muscle agonist like histamine to a surface receptor on the smooth muscle cell & subsequent rise in cytosolic Ca^{2+} concentration is the universal trigger for force generation in the smooth muscle. The increase in cytosolic Ca^{2+} does not occur in a spatially uniform manner. Rather, agonist exposure induces Ca^{2+} oscillations in SMCs which propagate as waves within the smooth muscle layer. These agonist-induced Ca^{2+} oscillations serve two critical functions in the smooth muscle: (1) the concentration/dose of agonist detected by the surface receptors is transduced into the frequency of Ca^{2+} oscillations with higher concentration of muscle agonist resulting in higher frequency of Ca^{2+} oscillations which can then be detected by downstream Ca^{2+} sensors & translated into dose-dependent increase in smooth muscle force. (2) Agonist induced Ca^{2+} oscillations can be transported from one SMC to its neighboring cell creating a Ca^{2+} wave that propagates around the circumference of the organ & enables synchronized contractions of SMCs necessary to constrict the airway/blood vessels. Agonist induced Ca^{2+} oscillations & long-range Ca^{2+} waves are therefore critical mechanisms that regulate vital parameters such as blood pressure & airway resistance. At present, very little is known about the role of extracellular mechanical factors such as ECM stiffness in regulating agonist-induced calcium oscillations & their transport across the many cells in a smooth muscle cell ensemble. **Findings**: in this study, we demonstrate a collective phenomenon in clusters of human airway smooth muscle, where ECM stiffening alters the intercellular communication between cells in an SMC ensemble causing these cells to increase their Ca^{2+} oscillation frequency & synchronize their Ca^{2+} oscillations. We show the altered intercellular communication causes SMCs in a confluent cluster to falsely perceive a higher dose of agonist & respond by generating a pathologically high level of force in response to a low dose of agonist. In contrast, isolated cells show no change in agonist response with matrix stiffening. We examined intercellular transport of Ca^{2+} in SMC cells & we show that contrary to dogma, the physical mechanism that enables intercellular calcium waves does not involve molecular transport across gap junctions or paracrine signaling through extracellular diffusion, but rather it is enabled by mechanical force-transfer

among cells in the cluster. **Conclusion:** Matrix remodeling in asthma & hypertension could be the cause & not the consequence of the disease progression.

P2424/B678

High-precision and Scalable Fabrication of Biomimetic Culture Environments for Enhancing Stem Cell Maturation.

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Stem cell technology holds great promise for properly modeling human biology in *vitro*. Building representative models relies on generating hierarchically organized cells and tissues. In *vivo*, this organization is driven by a complex interplay of cells and their environment, including the extracellular matrix (ECM). Traditional cell culture environments—typically composed of hard and unstructured glass or plastic— fail to fulfill the role of the ECM in development. Consequently, many in *vitro* stem cell models often fall short in correctly reproducing critical in *vivo* phenotypes because cultured cells oftentimes lose type-specific characteristics or express phenotypes indicative of an immature developmental stage. Considerable effort is directed at fabricating biomimetic culture environments that maintain or promote mature phenotypes. However, making biomimetic substrates typically involves costly or hard-to-reproduce techniques that are often incompatible with many standard assays. Our objective is to develop novel surfaces that mimic the mechanical and structural cues of the ECM. The fabrication scheme is based on high-precision photolithography techniques, and is thus highly reproducible, scalable, and amenable to integration with most industry-standard endpoint assays, including high-NA microscopy. We extended this approach to fabricate biomimetic culture surfaces out of elastomers that can be stretched in order to reproduce mechanical cues that are critical in the development and function of certain tissues. Our data demonstrate that various cell types are amenable to this approach. hiPSC-derived cardiomyocytes (CMs) showed in *vivo*-like myofibril alignment, sarcomere spacing and width, and expression of CM-specific proteins that are present in mature myocytes. Furthermore, higher-ordered 2D anisotropic myocyte tissues also showed adult-like structure and electrophysiological responses to drugs in *vitro* when compared to traditional unordered 2D isotropic constructs. Examples of phenotype enhancement of other adherent mammalian cell types will be presented, further demonstrating the utility of the approach for generating more representative cells and tissues. We conclude that our approach is a viable method for re-creating specific aspects of the ECM that are critical for driving the development and maturation of stem cells in culture.

P2425/B679

A High-throughput Hydrogel Array Platform for Investigating Stem Cell Mechanobiology.

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Extracellular matrix (ECM) mechanics and adhesivity regulate stem cell behavior in a complex, non-linear, and interdependent manner. To quantify these important regulatory relationships, there is a need for high-throughput matrix platforms that enable parallel culture of cells in many matrix conditions at one time. To this end, we developed a multi-well hyaluronic acid (HA) platform in which cells are cultured on combinatorial arrays of gels spanning a range of elasticities and adhesivities. Our approach is based on an orthogonal photopatterning strategy (Rape et al, *Nature Communications* 6: 8129)

previously developed in our laboratory, with the stiffness gradient implemented by a programmable light illumination system (Repina et al, *bioRxiv,* doi:10.1101/675892). Our new system allows individual treatment and analysis of each matrix environment while eliminating contributions of haptotaxis and durotaxis. Proof-of-principle study on human mesenchymal stem cells (hMSCs) validated that this platform can recapitulate expected relationships between matrix stiffness, adhesivity and cell spreading in hMSCs. C3H10T1/2 (also called 10T1/2) is a mouse embryonic cell line with functional similarities to mesenchymal stem cells with the potency to differentiate into white or brown adipocytes. Previously, we showed that mature white and brown adipocytes generate different degrees of contractility, which is associated with expression of distinct myosin isoforms. (Tharp et al. *Cell Metabolism* 27, 602). This finding raises the broader possibility that adipogenic efficiency may be sensitive to ECM mechanical cues. To test this hypothesis, we cultured 10T/12 cells in our platform and found that matrix stiffness strongly influenced the percentage of cells expressing adipogenic markers. Our work is among the first to quantify the relationship between 10T1/2 fate commitment and matrix stiffness. We anticipate that this platform has broad applications in dissecting molecular mechanisms of stem cell mechanosensing.

P2426/B680

2D Confinement Induces Shape Change and Increases Myosin Dependence of Zebrafish Epidermal Keratocyte Motility.

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Animal cells rely on actin-based motility in order to respond quickly to damage. Wound-healing fish keratocytes are one of the fastest cell types known, reaching speeds of several hundred nanometers per second. While the efficient motility mechanisms of keratocytes in culture has been studied and modeled extensively, we have recently demonstrated that keratocytes achieve similar speeds during wound healing in zebrafish embryos, despite exhibiting very different motility phenotypes. Compared to *ex vivo*, keratocytes in *vivo* migrate using more dynamic lamellipodia and actin structures, and their cell shapes are diverse and sometimes extremely elongated along the axis of migration. These cells are found basally in a bilayered epidermis during zebrafish development, so we sought to determine how the skin environment influences keratocyte motility, and what biomechanical and molecular processes are uniquely responsible for achieving rapid motility in skin. Keratocyte cell body height in the epidermis (~3 microns) is much smaller than in culture (~8 microns), so we hypothesized that confinement is a large factor in the phenotypic differences between in *vivo* and *ex vivo* keratocytes. Using an agarose overlay to confine isolated keratocytes, we found that confinement is sufficient to restrict keratocyte height to ~3 microns. 2D confinement causes other *in-vivo*-like phenotypes, including elongated cell shape and an increase in the amount and curvature of actin stress fibers. These observations have led us to conclude that physical confinement is likely to contribute significantly to the motility strategies adopted by in *vivo* keratocytes. We are developing genetic methods to directly test the effect of cell-cell interactions on keratocyte shape in the tissue environment. Although isolated keratocytes are still able to move under confinement, they exhibit reduced speed and persistence, as compared to unconfined cells. Keratocytes stop motility completely under dense agarose pads. Furthermore, confined cells, unlike unconfined cells, require activity of non-muscle myosin II for translocation. In the presence of high concentrations of blebbistatin, a myosin inhibitor, confined keratocytes form lamellipodia but cannot move their cell bodies. We are currently investigating models for the importance of myosin in confined keratocyte motility, including for traction force generation, lamellipodial actin turnover, and cell body

deformation.Taken together, our in *vivo and ex vivo* work will identify key interactions between motile cells and the layered epidermal environment.

P2427/B681

Triggering Receptor Expression on Myeloid Cells 1 Mediates Inflammatory Response to Low Shear Stress in a Three-dimensional Co-culture of Coronary Artery Smooth Muscle Cells and Endothelial Cells.

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Objectives: the main objective was to investigate the effect of different flow pattern on the expression of triggering receptors expressed on myeloid cells 1 (TREM-1) and its role in mediating inflammatory response on an in *vitro* model of human coronary artery. **Methods:** Human coronary artery smooth muscle cells (HCASMC) were mixed with neutralized type I collagen and plated on glass coverslips. The human coronary artery endothelial cells (HCAECs) were then plated on top of the collagen gels and cultured overnight. The co-culture was treated with or without low shear stress (LSS, 5 ± 3 dyne/cm^2), medium shear stress (MSS, 15 ± 3 dyne/cm^2), high shear stress (HSS, 30 ± 3 dyne/cm^2) with Flexcell Streamer for 4 h. Expression of TREM-1, cathepsin S and MMP-1 was examined by immunofluorescence (IF) staining, and mRNA of IL-1ß, IL-6, cathepsin S, and MMP-1 were quantified by real time qPCR. **Results:** LSS significantly stimulated TREM-1 expression, cathepsin S, and MMP-1, as evidenced by IF staining and mRNA expression (fold increase vs control) of IL-1ß (2.98 ± 0.11), IL-6 (7.96 ± 1.45), cathepsin S (3.65 ± 0.58), and MMP-1 (3.54 ± 0.85). Suppression of TREM-1 by siRNA resulted in significant inhibition on the IF expression of cathepsin S and MMP-1 proteins as well as mRNA transcripts (fold increase vs control) of the IL-1ß (1.62 ± 0.32, $p < 0.05$), IL-6 (1.25 ± 0.08, $p < 0.05$), cathepsin S (1.12 ± 0.13, $p < 0.05$), and MMP-1 (1.13 ± 0.23, $p < 0.05$). Similar outcomes on the inhibition of the inflammatory cytokines and enzymes were obtained with a pharmacologic inhibitor (LR-12) of TREM-1. Neither MSS nor HSS had significant effect on the expression of TREM-1 or aforementioned inflammatory mediators. **Conclusions:** These findings suggest that LSS promotes inflammatory response through TREM-1 expression in an in *vitro* model of human coronary artery tissue.

P2428/B682

Cell Adhesive Peptide Conjugated Chitosan Hydrogel as Salivary Gland Cell Culture Scaffolds.

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The structure is important for proper physical function, and should be replicated during tissue engineering for regenerative purposes. Extracellular matrix (ECM) provides suitable biochemical and structural support to the tissues in vivo and in vitro. The design of biomaterials mimicking the environment of the tissues is useful strategies, and many biomaterials mimic the structural and biological functions of ECM. Chitosan-based biomaterials have been demonstrated to be perform as salivary gland cell culture. Here, we crosslinked chitosan, a polysaccharide with many free amine groups, using dicarboxylic acids and conjugated ECM derived active peptides to the chitosan hydrogel. Ten dicarboxylic acids were examined to obtain the chitosan solution and found that seven liner saturated dicarboxylic acids could solve the chitosan to the water. Then, chitosan was crosslinked by dicarboxylic

acids with direct addition of N-hydroxy succinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (WSC). As results, glutaric acid ($HOOC(CH_2)_3COOH$), adipic acid ($HOOC(CH_2)_4COOH$), and pimelic acid ($HOOC(CH_2)_5COOH$) effectively generated transparent 3D hydrogels through the chitosan crosslinking. The stiffness of chitosan/dicarboxylic acids hydrogels are correlated with the length of dicarboxylic acids (glutaric acid > adipic acid > pimelic acid). Dicarboxylic amino acids of Glu and Asp were also tested. Chitosan/Glu solution exhibited SOL like liquid by addition of NHS and WSC and chitosan/Asp solution did not show the difference. Chitosan in mixed glutaric acid/Glu generated hydrogels by crosslinking and its stiffness was softened depending on the amount of Glu. These suggest that the stiffness of chitosan hydrogels can control by addition of Glu. Next, we conjugated ECM peptides to the chitosan hydrogel and examined its biological activities. The peptide-chitosan/dicarboxylic acid hydrogel effectively promoted human salivary gland cells (HSGs) attachment, seven days culture of HSGs on the peptide-chitosan hydrogel resulted differentiation and acinar like formation. The biological activities of peptide-chitosan/dicarboxylic acid hydrogel are different depending on the ECM derived active peptide. The peptide-chitosan/dicarboxylic acid hydrogel could use to analyze the cellular functions mediated by specific cell surface receptors and have the potential to use as cell culture matrices of HSGCs.

P2429/B683

The Evaluation of Extracellular Matrix Mimetic Elastin-like Polypeptide-laminin α1 Peptide.

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The extracellular matrix (ECM) is comprised of a large network of proteins that are not only essential for providing a physical scaffold but also the necessary biochemical and biomechanical signals for tissue development and repair. Laminins are a major component of the ECM and are responsible for a variety of biological processes such as cell adhesion, migration, proliferation and differentiation. Laminin α1 chain bioactive RGD containing peptide, A99 (AGTFALRGDNPQG), which was previously identified to promote strong cell attachment has demonstrated utility for tissue engineering applications. A variety of biomaterials have been used as scaffolds for these bioactive peptides, but many are not suitable for various reasons such as scalability, harsh reaction conditions or lack of homogeneity. Here, we are the first to describe methods for preparing chemically conjugated and recombinant A99 to elastin-like polypeptides (ELPs) as the scaffold and characterize their behavior as well as evaluate the coating efficiency, cell attachment and spreading activity. Preliminary evidence suggest that the A99-ELP fusion construct has great potential for use as a biomaterial in cell culture applications as they greatly promote cell adhesion and spreading. ELPs are biocompatible protein-polymers that are thermo-responsive and self-assemble in response to heat. Below a lower critical solution temperature (LCST), they are highly soluble but above the LCST, ELPs phase separate, forming coacervates. They provide an excellent structural framework for bioactive peptides and their intrinsic biophysical characteristics allow for simple production with high yields and purity making them an ideal biomaterial for tissue engineering.

P2430/B684

Characterization of Dystroglycan Binding in Adhesion of Human Induced Pluripotent Stem Cells to Laminin-511 E8 Fragment.

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Human induced pluripotent stem cells (hiPSCs) are promising cells for regenerative medicine, similar to human embryonic stem cells (hESCs). These cells have an infinite proliferative potential and capacity for differentiation into all cell types of the body. They are prospective cell sources for applications such as transplantation therapy and drug discovery. The ability to stably expand the stem cells is a fundamental technical requirement for these applications.Stem cells were originally maintained in complex culture systems, comprising a mouse feeder cell layer, media containing foetal bovine serum, an extracellular matrix-rich environment for cell adhesion, and soluble growth factors. Many groups have tried to optimize xeno-free culture conditions for stem cells. In the development of cell culture systems, a fragment of laminin-511 (LM511-E8) was found to improve the proliferation of stem cells. LM511-E8 commercially available as iMatrix-511, is currently used as a culture substrate in combination with various defined medium systems. In human stem cells, integrin α6β1 is a major isoform at cell surfaces. The fragment containing the integrin α6β1-binding site, enables superior adhesion of single cell-dissociated cultures of hESCs and hiPSCs. Therefore, it is well-known that the adhesion of hiPSCs to LM511-E8 is mainly mediated through integrin α6β1. However, the involvement of non-integrin receptors remains unknown in stem cell culture using LM511-E8. In this study, we show that dystroglycan (DG) is strongly expressed in hiPSCs. The fully glycosylated DG is functionally active for laminin binding, and although it has been suggested that LM511-E8 lacks DG binding sites, the fragment does weakly bind to DG. We further identified the DG binding sequence in LM511-E8, using synthetic peptides, of which, hE8A5-20 (human laminin α5 2688-2699: KTLPQLLAKLSI) derived from the laminin coiled-coil domain, exhibited DG binding affinity and cell adhesion activity. Deletion and mutation studies show that LLAKLSI is the active core sequence of hE8A5-20, and that, K2696 is a critical amino acid for DG binding. We furtherexplored the possibility that DG-binding peptides could serve as culture substrata. Our results show that hiPSCs adhere to hE8A5-20-conjugated chitosan matrices. The amino acid sequence of DG binding peptides would be useful to design substrata for culture system of undifferentiated and differentiated stem cells.

P2431/B685

Measuring the Inhibitory Effects of *Pseudomonas Aeruginosa* on *Candida Albican*'S Dimorphic Switch.

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Candida albicans (Ca) and *Pseudomonas aeruginosa* (Pa) are commonly found together in polymicrobial lung infections that occur in patients with cystic fibrosis and in immunocompromised individuals. Both microbes are opportunistic pathogens, which display remarkable phenotypic plasticity and are known to have bidirectional antagonistic interactions. Much of what has been learned about Ca and Pa interactions rely on traditional in *vitro* co-culturing methods. While the quorum sensing molecules (QSMs) that mediate Ca and Pa communication were first identified with standard culturing methods, there are still many basic questions about how these two organisms interact and contribute to

infections. For example, liquid cocultures typically have >10^6 cells, which makes it difficult to quantify phenotypic changes (e.g. yeast to hyphal transition) overtime. An other challenge is to differentiate effects that require physical contact between Ca and Pa versus those that solely depend on QSMs. Here, we use a newly developed microfluidic platform that allows for coculturing two or more species permitting cross-communication either in adjacent separate hydrogels or well-mixed within one hydrogel. Ca and Pa cells are grown in hydrogels within a microfluidic device that permits cross-communication (e.g. diffusion of QSMs) under different nutrient and environmental conditions. Simultaneously, we can image both populations of Pa and Ca cells in real-time to quantify phenotypic changes such as the hyphal transition in Ca or changes in Pa motility. As a first test of our system, we are measuring the inhibitory effects of Pa on Ca hyphal development under different nutrient conditions. Our initial results show that nearly 100% of individual Ca cells develop hyphae in the hydrogels at 37°C. When a hydrogel carrying Pa is positioned adjacent to the Ca hydrogel we have not observed an inhibition in hyphal development. Yet, if we mix Pa and Ca together we observe a significant decrease in hyphal Ca cells suggesting that physical contact between Pa and Ca might be required to inhibit the yeast-to-hyphal switch, which will be further investigated. This work sets the stage to measure more complex interactions not only between Ca and Pa but potentially in studies of polymicrobial interactions that far exceed two species.

P2432/B686

Low Adhesive Scaffold Collagen Prepared from Type I Collagen Promotes Neuronal Survival and Neurite Outgrowth.

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[Objective] the failure of damaged adult CNS axons to regrow results in permanent disabilities with spinal cord injury. In adults, spinal nerve axons rarely regenerate after injury. Pepsin-hydrolyzed type I collagen commonly uses for spinal cord injury repair as a scaffold. However, the transplanted collagen is not the most suitable for spinal cord repair as similar as scar. The poor regeneration of mature axons is attributed to complicated intrinsic and extrinsic mechanisms. Therefore, the repair and regeneration of spinal cord is a very attractive research for tissue engineering and regenerative medicine. Recently, we succeeded in developing low adhesive scaffold type I collagen from porcine skin (LASCol). The cells on the LASCol-coated dish collide each other one after another and get to form spheroid larger. In this study, we report that LASCol has beneficial effects on neuronal survival and axonal outgrowth. [Methods] Culture dish was coated with LASCol, Atelocollagen (Cellmatrix Type I-C, Nitta Gelatin Inc.), or poly-L-lysine. Rat neurons were prepared from the hippocampus of postnatal day 1-2 (P1-2) SD rats. Neurons were seeded and cultured on each coated-dish with Neurobasal Medium including B27 supplement. To investigate neuronal survival, we observed appearance of neurons by a phase-contrast microscope. To evaluate neural regeneration, we stained neurite outgrowth with anti-beta tubulin antibody. Moreover, morphology of regenerated neurite outgrowth was observed by scanning electron microscopy. [Results] Rat neurons strongly adhered on the LASCol coated-dish as comparison with other two coated-dish groups. On the LASCol coated-dish, beta-tubulin of neurons significantly expressed than other experimental group. Moreover, scanning electron micrograph demonstrated that neurite of each neuron form long and fine shape by culturing on LASCol matrix. [Summary and Conclusions] We demonstrated that LASCol supports neuronal survival and axonal regeneration. [Funding and

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P2433/B688

Characterizing the Biophysical Properties of a Tunable Hydrogel System for 3D Cell Culture.

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The hydrogel-based 3D cell culture model more accurately depicts the microenvironment of cells in *vivo* than the traditional 2D cell culture system, which enables cells to grow, develop, operate, and produce feedback as if they were in a living organism. As we continue to learn more about cell growth in 3D microenvironment, we come to understand that, similar to the 2D cell culture system, the cell-cell communication and cell-matrix interactions rely on many variables of the hydrogel biophysical properties including stiffness, density, porosity, binding ligands, and composition. However, because the current animal-derived hydrogel systems struggle with the batch-to-batch consistency, while the final properties of most hydrogel systems are depended on several key factors such as culture medium, cell density, and supplement compounds; there is a lack of a thorough study on the biophysical properties of current hydrogel-based 3D cell culture products. Here, we used a xeno-free, tunable hydrogel system, VitroGel, to characterize the hydrogel rheological properties in response to ionic molecules, compositions of cell culture mediums, and supplements such as glucose and serum. The tests cover different stages of hydrogel (formation, stable, and injection) at various dilution and mixing ratios. The molecular diffusion properties of hydrogels at different concentrations and formation states were also characterized with phenol red, trypan blue, BSA and IgG. The glioblastoma cancer cells (U87 MG, SNB 75, et al) were cultured in the 3D hydrogel to evaluate the intercellular characters in response to the different hydrogel conditions. The data indicated that the VitroGel system can be a platform with defined and adjustable biophysical properties to support drug screening and toxicology studies, tissue engineering, regenerative medicine, and other basic cell biological studies.

P2434/B689

3D Printed Tumor Spheroids for Disease Modeling and Chemotherapeutic Drug Screening.

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3D Printed Tumor Spheroids for Disease Modeling and Chemotherapeutic Drug Screening Abstract 3D bioprinting is intended for regenerative medicine and disease model development for drug discovery in-vitro. Herein, we evaluated the printability of a series of xeno-free and ready-to-use bioinks, developed from the tunable hydrogel (VitroGel) system. We observed that Ink H4 and Ink H4-RGD hydrogel displayed excellent rheological properties among all screened samples. Ink H4-RGD was printable with less cell destructive extrusion pressure and showed more than 90% cell viability for both tumor and normal cells. Oscillatory rheological measurements of Ink H4-RGD hydrogel showed good printability with a wide temperature range of 20 and 37^{o}C. The constructed cell-laden scaffold maintained its stiffness for more than 20 days of incubation at 37^{o}C. NSCLC PDX (EGFR T790M) cells, which were cultured in 3D printed Ink H-RGD scaffold, showed high cell proliferation with tumor microenvironment and spheroid formation (500 µm in diameter) within 7 days as analyzed by NucBlue/Actin green and E-

cadherin immunofluorescence staining. IC50 values of docetaxel, doxorubicin and erlotinib demonstrated higher resistance in 3D spheroids of NSCLC-PDX, MDA MB231 WT and HCC B-27 cells when compared to 2D monolayers cells, as analyzed by cytotoxicity assay ($P < 0.001$). Our results of the rheological analysis, shape fidelity, scaffold stability and biocompatibility of Ink H4-RGD suggest that it could be considered for cell printing and soft tissue development for high throughput screening of various anti-cancer drugs.

P2435/B690

Ability of Individual Cells to Remodel Local Substrate Fibers Guides Their Polarization and Migration Persistence.

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Directing cell migration is critical for both tissue engineering/regeneration and controlling cancer metastasis. Cell migration has been guided directly via physical structures and gradients. However, cell migration control is also an emergent property of fibrous extracellular matrix (ECM) and scaffolds in which cells can rearrange fibers to generate their own guidance cues. This positive feedback system in which cells simultaneously follow and align fibers has typically been studied in the context of multi-cell masses and large-scale, permanent ECM deformations. However, such systems do not accurately recapitulate single, autonomous cell invasion. Here, we asked whether the positive feedback afforded by flexible substrate fibers influences individual cell migration decisions. HT-1080 fibrosarcoma cells were seeded as spheroids into 3D collagen type I matrices that were photocrosslinked or non-crosslinked ("native"). Cells invaded from the spheroids into the matrix as individuals and their migration paths were tracked over several hours. Cells in crosslinked matrices showed a decrease in fiber reorganization and alignment, and a concomitant decrease in migration persistence relative to cells in native matrices, suggesting that an individual cell's ability to align fibers affects is persistence. Parallel experiments were conducted in which spheroids were instead seeded onto 2D substrates with rigid polycaprolactone (PCL) fibers of varying densities and on glass. Cell persistence increased with increasing fiber density, and was highest on isotropic glass, suggesting that a fibrous substrate structure can limit migration if it cannot be aligned by cells. These findings indicate that the autonomous migration of individual cells in fibrous environments is affected by the fibers' responsiveness to remodeling and suggest a means to influence cell invasion by modulating cells' ability to generate their own contact guidance cues.

Cell Death: Apoptosis

P2436/B692

Compulsive Methamphetamine Taking and Abstinence Under Punishment Are Associated with Differential Expression of Atf2-p53 in the Rat Dorsal Striatum.

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Addiction to methamphetamine (METH) affects approximately 25 million people in the world. Our laboratory has focused its attention on deciphering the molecular neurobiology of compulsive METH taking behaviors by using the drug self-administration (SA) model. Towards that end, we have used a reproducible METH SA model accompanied with contingent footshock punishment to dichotomize METH self-administering Sprague-Dawley rats into two phenotypes. Some animals continue to self-

administer METH compulsively (shock-resistant, SR) whereas others decrease their consumption (shock-sensitive, SS) in the presence of punishment. This model thus includes two DSM criteria of addiction, namely escalated drug intake and continuous use in the presence of adverse consequences. In the present study, we have found that compulsive METH taking (resistant) rats exhibited increased striatal abundance of phosphorylated activating transcription factor 2 (ATF2) in comparison to shock-sensitive (sensitive) rats. ATF2 is a histone acetyltransferase that adds acetyl groups to histones H2B and H4. Once active via phosphorylation, ATF2 can homodimerize or heterodimerize with other AP1 transcription factors in responses to a diverse set of cellular responses that are involved in inducing apoptosis. We thus reasoned that downstream death cascades might be activated in the shock-resistant METH rats. To test this idea, we measured the expression of the apoptotic protein, p53, and found significant increases in p53 protein levels in the dorsal striatum of resistant METH SA rats in comparison to other groups. Increased p53 was accompanied by decreased expression of the p53 target protein, Bcl-2. These results indicate that compulsive METH taking may be associated with the existence of neuropathological changes like those reported in post-mortem human brains.

P2437/B693

Calpain 1-mediated MST1 Activation Regulates NMDA-induced Excitotoxicity in Cortical Neurons.

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Overstimulation of the ionotrophic N-methyl-D-aspartate (NMDA) receptor may cause an excessive influx of calcium that leads to excitotoxic cell death. In this study, we identified MST1 (mammalian sterile 20-like kinase-1), a serine/threonine kinase known to promote apoptosis, as a novel substrate of calpain 1. We showed that induction of excitotoxic stress in primary cortical neurons by treatment with NMDA resulted in the cleavage and activation of MST1, both of which are dependent on calpain 1 protease activity. Calpain 1-mediated cleavage promotes the nuclear translocation of MST1 resulting in the phosphorylation of histone 2B as well as neuronal cell death. Taken together, our findings suggest that NMDA induces the stimulation of MST1 in a calpain 1-dependent manner and that this stimulation is an integral part of the mechanism for NMDA-induced excitotoxicity.

P2438/B694

Structural and Spatio-temporal Organisation of Bax and Apaf1 during Apoptosis.

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Apoptosis involves a series of highly regulated events towards cell death. Upon apoptotic signals, the protein Bax inserts into the outer mitochondrial membrane (OMM), assembles into clusters and ruptures the OMM. This leads to release of cytochrome c from the intermembrane space into the cytosol, where it interacts with Apaf1 forming the apoptosome complex that activates caspases. Although the sequence of protein functions is known, their spatial organisation remains elusive. The role of Bax clusters, their composition and structure are enigmatic. Apoptosome formation and distribution is also unknown. Here we address the organisation of Bax clusters and the apoptosome component Apaf1, in particular their structure, dynamics and interactions in human cells. By cryo-electron tomography (ET) of focused ion-beam milled cells, we previously showed that Bax clusters are irregular meshwork, similar to sponge-like structures, possibly contributing to OMM rupture formation by

sequestering OMM components. To explore whether Bax clusters serve as compartments for downstream apoptotic events such as apoptosome formation, we used Bio-ID and immunofluorescence. We found that the mitochondrial fission factors DRP1 and MFF are part of Bax clusters, whereas Apaf1 is not. These results suggest that Bax clusters promote mitochondria reorganisation, but do not host apoptosome formation. By fluorescence imaging, we observed that Apaf1 forms transient foci that disassemble shortly before Bax clusters appear. By ET of resin-embedded cells, we found that Apaf1 foci are dense cytoplasmic structures intersected with ribosomes, thereby differing from Bax clusters which are amorphous, ribosome-excluding zones in resin-embedded cells. We are now investigating the structure of Apaf1 foci by cryo-ET. Whether Apaf1 foci are sites of apoptosome pre-assembly remains to be determined. Because of their occurrence before Bax clusters, Apaf1 could potentially be sequestered into foci to avoid apoptosis until cell fate is determined by cytochrome c release. Based on our data, we propose that the apoptotic pathway is not only organised in time, but also uses special environments such as Bax clusters and Apaf1 foci to regulate cell death. This spatial and structural compartmentalisation could allow to locally disturb cell and organelle processes, and help fine-tuning cell fate.

P2439/B695

Manuka Essential Oil Decreases the Proliferation of Human Cancer Cell Lines.

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Since research on the effects of essential oils on human cell lines is limited, the goal of this project was to treat cancer cells lines with manuka essential oil at different concentrations and ascertain the effects on cell proliferation. The two cancer cell lines tested were fibrosarcoma (HT-1080) cells and cervical adenocarcinoma (HeLa) cells. Manuka oil is popular in many skincare products because of its antibacterial and antiinflammatory properties that treat several skin conditions. However, manuka oil also contains an active ingredient that is commonly found in herbicides and is potentially toxic to human cells at certain concentrations. To conduct the experiment, both cell lines were grown on 24-well plates and subconfluent cultures were treated with varying concentrations of manuka oil for 24 hrs. The effect of the oil on proliferation was measured through direct cell counting using trypan blue dye exclusion and through the use of an MTT assay. A trend was found that as the concentration of oil increased, viability of both cell lines decreased. Fibrosarcoma (HT-1080) cells treated with 500 µg/ml manuka oil had significantly decreased proliferation, with a 55% decrease in viable cells compared to the control. MTT assay results also reflected this trend, with a significant decrease in MTT activity seen in cells treated with 250 and 500 µg/ml manuka oil. Increasing amounts of manuka also decreased proliferation of HeLa cells, with 500 µg/ml manuka oil resulting in a 46% decrease in viable cells compared to the control. To determine if the decreased cell number is the result of apoptosis, PARP cleavage assays will be performed. Western blotting will be used to quantify MAPK proteins usually expressed as part of cell stress responses.

P2440/B696

A New Paradigm for Regulation of Cell Death by Intracellular Ph.

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Regulated cell death is essential during development to precisely pattern tissues and avoid developmental errors. Dysregulation of cell death is associated with pathologies including cancer

(reduced cell death) and neurodegeneration (increased cell death). Dysregulated intracellular pH (pHi) dynamics are also associated with these diseases, where cancer cells have constitutively higher pHi than normal cells, while degenerating neurons have lower pHi. Supporting this, in vitro experiments showed that the apoptotic caspase enzymes showed increased activity at low pH. Together, these observations led to the current view that cell death is enhanced at low pHi, and inhibited at higher pHi. Our objective in these studies is to directly test this prediction in vivo. We used transgenic Drosophila lines that overexpress the Na-H exchanger *DNhe2* specifically in the eye (*GMR>DNhe2*), which increases pHi and resulted in a smaller, mispatterned adult eye. We performed cell counts in pupal eyes midway through metamorphosis. We found a significant decrease in the number of interommatidial lattice cells at the end of pattern formation from an average of 15 cells in control to 11.4 cells in *GMR>DNhe2*. We performed temperature shift experiments to temporally regulate transgene expression, and determined that the critical phase for expression of *DNhe2* is pupal eye development, which coincides with two well-studied waves of apoptotic cell death. We next tested for genetic interactions between *DNhe2* and apoptotic genes. We found that the pH-dependent cell death is p53-dependent but caspase-independent, which is inconsistent with apoptosis, but suggests autophagy. We are currently testing whether molecular markers for autophagy are increased in *GMR>Dnhe2* eyes. Together, our findings will elucidate mechanisms for pH-regulation of conserved, critical developmental processes and provide evidence for new paradigms in growth control.

P2441/B697

Elucidate BaxΔ2-casapse 8 Interaction Using Computational Modeling.

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BaxΔ2 is a functional pro-apoptotic Bax isoform. Instead of targeting mitochondria, BaxΔ2 proteins form cytosolic aggregates and activate caspase 8. Previous experimental studies indicated that the C-terminal helix α9 structure of BaxΔ2 is critical for caspase 8 recruitment. However, the interaction between these two proteins at the molecular level is unknown. In this study, molecular dynamics (MD) simulations were performed on BaxΔ2 and caspase 8 binding-deficient mutant (L164P, located in the helix α9). Protein-protein docking was also used to estimate the binding affinities of BaxΔ2 and L164P mutant with caspase 8. We observed that the movements of BaxΔ2 and L164P mutant were different during MD simulations. The results from protein-protein docking showed that helices α9 of both BaxΔ2 wild type and mutant were near the caspase 8 binding site. However, only wt H9, but not mt H9, forms hydrogen bonds with caspase 8. These results suggest that BaxΔ2 is more favorable for binding to caspase 8 than L164P mutant. Together, these data provide a structure basis for explaining the BaxΔ2 function in Caspase 8-dependent cell death.

P2442/B698

High-glucose Diet Reduces Fertility in *C. Elegans*: a Role for Apoptosis.

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The amount of sugar in the average human diet has increased dramatically over the past several decades. Increased dietary sugar is correlated with increases in metabolic diseases such as Type 2 diabetes and obesity; these diseases, in turn, affect a range of biological processes, including fertility. C. elegans is an excellent model organism for studying the effects of high-sugar diet on fertility: 60-80% of genes in the human genome have an ortholog in C. elegans. Among these conserved genes are those

that control processes such as insulin signaling, glucose storage, and oocyte aging. Data from our lab and others has shown that a high-glucose diet leads to decreased fertility in *C. elegans* hermaphrodites, but the mechanisms that cause this decrease are unknown. Many types of stressors, including starvation, DNA damage, and pathogen exposure, decrease fertility by increasing germline apoptosis. We hypothesized that a high-glucose diet may also act as a stressor that decreases fertility via an increase in germline apoptosis. We find that in the absence of the core apoptosis proteins CED-3 (caspase 9 ortholog) and CED-4 (Apaf1 ortholog), high-glucose diet does not significantly reduce fertility, consistent with our model. Next, we investigated whether cellular pathways that are known to activate apoptosis are implicated in the fertility decrease seen on a high-glucose diet. We find that the CEP-1 (p53) DNA damage pathway is not required for the response to high-glucose diet: in the absence of CEP-1, a high-glucose diet reduces fertility to the same extent as it does in wild-type animals. We are currently investigating whether other known pathways, like the EGL-1 pathogen response pathway, are activated by a high-glucose diet to reduce fertility. Through this work we hope to understand the cellular consequences of a high-glucose diet for gamete survival and quality and the genetic pathways that regulate those responses.

P2443/B699

The Role of the C-type Lectin Receptor DC-SIGN in the Survival of Lymphoblastic B Cells.

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C-Type Lectin Receptors (CLR) are soluble or membrane associated proteins capable of binding to carbohydrate ligands such as beta-glucans and mannans. While membrane-associated CLR can function as cell adhesion molecules, in immune cells they also function as a Pattern Recognition Receptors (PRR) mediating innate immune responses. Due to their high avidity for glycoproteins and glycolipids-associated carbohydrates, CLR recognize many pathogens including bacteria, fungi and viruses. DC-SIGN is a type-II transmembrane CLR expressed primarily dendritic cells (DC) and to a lesser extent in other antigen presenting cells like macrophages and activated B cells. DC-SIGN recognizes mannose and fucose bearing glycans and it functions as a receptor for several viruses (e.g. Zika, Sindbis, Dengue and HIV-1). In dendritic cells, DC-SIGN ligation leads to the recruitment and activation of scaffolding and signaling proteins LSP1, KSR1, CNK and Raf-1 modulating cytokine expression and cell function. Similar to DC, activated B cells also express DC-SIGN, however, the role of this CLR in B cell function is still elusive. In B cells, DC-SIGN binds to the HIV-1 envelope protein gp120 and while this interaction promotes T cell trans-infection, the signaling events triggered in response to gp120 binding and their outcome in B cell function is unknown. Our main goal is to identify and characterize signaling pathways in human B cells in response to DC-SIGN activation. To this end, we used two different lymphoblastic cell lines that constitutively express DC-SIGN as a model to study DC-SIGN-mediated signaling. Our preliminary observations showed that DC-SIGN expression sensitizes these cells to apoptosis and DC-SIGN activation using the HIV-1 protein gp120, mannan or an anti-DC-SIGN specific antibody reduced this phenotype. By using an antibody array and immunoblotting, we identified changes in the expression of proteins involved in cell survival and apoptosis including BclXL, Bcl-2 and Bim. Interestingly, treating lymphoblastic B cells with cytarabine in the presence of mannan or anti-DC-SIGN reduced the pro-apoptotic effect observed with cytarabine alone confirming our observations of DC-SIGN-mediated survival. Finally, RNA sequencing analysis of lymphoblastic B cells treated with the DC-SIGN ligand mannan showed the transcriptional upregulation of genes involved in lipid biosynthesis and mitochondrial metabolism. Our study suggest that activation of DC-SIGN in lymphoblastic B cells causes

the upregulation of anti-apoptotic genes promoting cell survival, which correlates with changes related to mitochondrial biology and lipid metabolism. These results suggest a novel role for DC-SIGN in the physiology of lymphoblastic B cells and potentially B cell lymphomas.

Autophagy

P2444/B700

Molecular Mechanisms of Atg-9 Localization at Synapses.

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Autophagy is an evolutionarily conserved cellular degradation process in which cytosolic components are engulfed by double membrane structures known as autophagosomes. ATG-9 is the only known transmembrane protein that is a core component of the process. It is thought to cycle between immature autophagosomes and other organelles, and is important for autophagosome formation. Previous work from our lab demonstrated that ATG-9 localizes to presynaptic regions and is associated with autophagosome biogenesis at the synapse (Stavoe and Hill et al., 2016). How ATG-9 localizes within presynaptic regions and traffics between membranes to regulate autophagy is not well understood. To identify molecules required for ATG-9 localization to synapses, we performed candidate screens in AIY. We have identified four endocytic mutants, *unc-26/Synaptojanin 1*, *unc-57/Endophilin A*, *unc-11/AP180* and *dyn-1/dynamin-1*, which display highly penetrant redistribution of ATG-9 to purported subcellular compartments at presynaptic regions. We found that mutants of exocytosis regulators unc-13/Munc13 and unc-18/Munc18 suppressed the ATG-9 phenotype in *unc-26/Synaptojanin 1* mutants, suggesting a role for exocytosis in mediating ATG-9 localization at the synapse. To identify ATG-9 subcellular compartments, we have performed candidate screen looking for mutants that phenocopy endocytic mutants and colocalization study. We tested candidate autophagy mutants and found that mutants defective for early stages of autophagy phenocopied endocytic mutants, suggesting that ATG-9 subcellular compartments can be early autophagic structures. In addition, ATG-9 colocalizes with clathrin heavy chain in both wild type and *unc-26/Synaptojanin 1* mutants, and partially colocalizes with synaptic vesicle proteins, suggesting that ATG-9 accumulates to clathrin-coated structures in the mutants. We hypothesize that ATG-9 cycles between plasma membrane and immature autophagosomes at synapses, and that this process is regulated by clathrin-mediated endocytosis.

P2445/B701

Ipmk Mediates Activation of Ulk Signaling and Transcriptional Regulation of Autophagy Linked to Liver Inflammation and Regeneration.

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Autophagy plays a broad role in health and disease. Here, we show that inositol polyphosphate multikinase (IPMK) is a prominent physiological determinant of autophagy and is critical for liver inflammation and regeneration. Deletion of IPMK diminishes autophagy in cell lines and mouse liver. Regulation of autophagy by IPMK does not require catalytic activity. Two signaling axes, IPMK-AMPK-Sirt-1 and IPMK-AMPK-ULK1, appear to mediate the influence of IPMK on autophagy. IPMK enhances

autophagy-related transcription by stimulating AMPK-dependent Sirt-1 activation, which mediates the deacetylation of histone 4 lysine 16. Furthermore, direct binding of IPMK to ULK and AMPK forms a ternary complex that facilitates AMPK-dependent ULK phosphorylation. Deletion of IPMK in cell lines and intact mice virtually abolishes lipophagy, promotes liver damage as well as inflammation, and impairs hepatocyte regeneration. Thus, targeting IPMK may afford therapeutic benefits in disabilities that depend on autophagy and lipophagy-specifically, in liver inflammation and regeneration.

P2446/B703

Molecular Crosstalk between Autophagic and Endo-lysosomal Pathways Regulates Cellular Clearance in the Retinal Pigment Epithelium.

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Interactions between photoreceptors and the retinal pigment epithelium (RPE) - including the visual cycle, outer segment phagocytosis and glucose transport - are essential for maintaining vision. Efficient lysosomal degradation of phagocytosed outer segments and other cellular debris is critical for RPE health. Autophagy proteins participate in LC3-associated phagocytosis of outer segments and in classical autophagic degradation within the RPE. We have found that hierarchical recruitment of autophagic machinery to outer segment phagosomes modulates membrane dynamics and leads to transcriptional activation of lysosome biogenesis. Our work reveals a highly coordinated process that links LC3-associated phagocytosis to scaling RPE degradative capacity to ensure efficient clearance of cellular debris and phagocytosed outer segments.

P2447/B704

ALS/FTD Mutations in UBQLN2 Impede Autophagy Due to a Reduction in Autophagosome Acidification.

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Missense mutations in UBQLN2 cause X-linked dominant inheritance of amyotrophic lateral sclerosis with frontotemporal dementia (ALS/FTD). UBQLN2 is one of four UBQLN proteins expressed in humans that regulate proteostasis. The proteins facilitate clearance of misfolded proteins through the proteasome and autophagy degradation pathways. Interestingly, immunostaining of neuronal tissue from human UBQLN2 cases and UBQLN2 mouse models of ALS/FTD have revealed major disturbances in the autophagy marker p62, suggesting ALS/FTD mutations in UBQLN2 may disturb autophagy. However, neither the role of UBQLN2 in autophagy nor how ALS/FTD mutations affect autophagy is known. Here we describe results of the effects of CRISPR/Cas9 inactivation of UBQLN2 on autophagy and of the effects of expression of wild-type and ALS/FTD mutant UBQLN2 proteins in cells and mouse. Cells disrupted in UBQLN2 expression had major defects in autophagic flux, including reduction in acidification of autophagosomes. This acidification defect was discovered through use of a dual split-Venus reporter system comprising tandem-tagged C-Venus-mCherry-LC3 and N-Venus-UBQLN2 reporters. Transfection of these constructs into cells resulted in labeling of autophagosomes with both Venus and mCherry fluorescence, whereas autolysosomes were labeled only by mCherry fluorescence. The reconstitution of Venus fluorescence in autophagosomes demonstrates UBQLN2 can localize to autophagosomes. We next determined whether the autophagosome acidification defect in UBQLN2 knockout cells could be rescued by expression of WT or ALS/FTD mutant UBQLN2 proteins. Our results

show that the acidification defect can be rescued by expression of WT UBQLN2, but not by any of the 5 different ALS/FTD mutant UBQLN2 proteins tested, suggesting the ALS mutations causes loss-of-function. To identify the underlying reason for the acidification defect we examined the proteome of neuronal tissue from ALS/FTD mutant mice to determine whether expression of any subunit of the vacuolar ATPase pump was altered. This analysis revealed a specific reduction in ATP6v1g1, a subunit of the vacuolar ATPase pump, only in transgenic mice expressing a P497S UBQLN2 mutation but not in mice expressing WT UBQLN2. Diminished accumulation of the same subunit was also found in cells and mice disrupted of UBQLN2 expression, suggesting that the reduction of the subunit stems from loss of UBQLN2 function. Taken together our results suggest that ALS/FTD mutations in UBQLN2 drive pathogenesis through loss of function in autophagy through a reduction in expression of the catalytic ATP6v1g1 subunit. Efforts to restore proper expression of the subunit may provide therapeutic opportunities to treat ALS/FTD caused by UBQLN2 mutations.

P2448/B705

Cytotoxicity of Arginine Deprivation to Acute Myeloid Leukemia Cells Is Mediated by Autophagy.

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A large number of tumor types have been shown to be auxotrophic for arginine, secondary to the loss of expression of the urea cycle enzyme argininosuccinate synthetase-1 (ASS1), hence sensitive to arginine deprivation using arginine degrading enzymes. We and others have shown that Acute Myeloid Leukemia (AML) cells are auxotrophic for arginine and sensitive to a recombinant, cobalt-substituted human arginase I [HuArgI (Co)-PEG5000]. However, the impact of arginine deprivation on the flux of autophagy, in addition to the potential contribution of autophagy to the observed cell death in AML have not been investigated yet. In this study, we assess the activation of autophagy following arginine deprivation, in five AML cell lines, and determine the impact of autophagy on cell cytotoxicity through its inhibition using the downstream autophagy inhibitor chloroquine (CQ). [HuArgI (Co)-PEG5000]-induced arginine deprivation led to a marked and sustained activation of autophagy, in AML cell lines, starting at 12 hours and lasting up to 120 hours following arginine deprivation. Addition of exogenous L-citrulline, which reverses arginine dependence in partially auxotrophic cells, led to the complete reversal of the activation of autophagy in partially auxotrophic cell lines, demonstrating that the activation of autophagy is induced by arginine deprivation. Inhibition of autophagy led to a significant decrease in cell death, reaching complete resistance at late time points (between 72 and 120 hours), indicating that cell death following arginine deprivation is mediated by the activation of autophagy (death by autophagy). Addition of the ROS scavenger N-acetylcysteine (NAC), repressed the autophagic response and reversed cytotoxicity of arginine deprivation in AML cells, indicating that the activation of autophagy, and subsequent cell death, following arginine deprivation, occur secondary to ROS generation.We have shown that [HuArgI (Co)-PEG5000]-induced arginine deprivation in AML cells leads to extensive and sustained activation of autophagy which, in turn, leads to the induction of cell death under prolonged arginine deprivation.

P2449/B706

Glyceraldehyde-3 Phosphate Dehydrogenase Bridges the Host Defense Peptide Autophagic Activity to Limit Intracellular Pathogen.

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Antimicrobial peptides (AMPs) are major effectors of the innate immune system. They are mainly expressed by epithelial and immune cells. Involved in many critical cellular functions like their ability to kill pathogens via autophagy and immunomodulation, reflects their anti-infective mode of action. *Mycobacterium tuberculosis* (*Mtb*), the causative agent of human tuberculosis (TB), has bedeviled humans for many years. Inside macrophages, *Mtb* resides within phagosomes, where its survival is subjected to the way it alters the phagosomal environment by inhibition of phagolysosome fusion and acidification and thereby getting ingress to the cytosol. Host-directed therapy (HDT) for TB treatment is a passable concept under which host response is targeted, like alteration of host inflammatory pathways or augmentation of cellular anti-microbial defense system. Successful host cell strategy against *Mtb* is its clearance during early stages of infection. During these initial stages, pulmonary TB patients showed upregulation of glycolysis gene glyceraldehyde-3 phosphate dehydrogenase (GAPDH) and an enhanced glycolytic flux in host immune cells. Host cell metabolic reprogramming occurs to mount effective host antimicrobial and immune response against *Mtb* infection. The present study thus attempts to explore cellular and molecular effects of GAPDH on host defense peptide antimicrobial actions. We investigated the host cell GAPDH dependent roles of LL-37 peptide in *Mtb* infected macrophages. We found that peptide activates autophagy and promote the co-localization of *Mtb*-containing phagosomes with lysosomes. We initially determined that silencing GAPDH abrogated LL-37 mediated decrease in intracellular viability of *Mtb*. GAPDH knockdown cells also failed to induce autophagosome machinery upon peptide treatment. Finally, we assessed that GAPDH knockdown cells were inefficient to activate LL-37 mediated P38 MAPK and calcium/calmodulin-dependent protein kinase cascade in infected macrophages. In our current work we also investigated the role of cell surface recruited GAPDH as a putative receptor for LL-37 peptide. Co-immunoprecipitation analysis revealed this peptide interacts with GAPDH localized in membrane of macrophages. Using confocal microscopy based analysis we found specific colocalization of LL-37 peptide with GAPDH on cell surface and also in the same endocytic compartment inside cell. Collectively our findings suggests that anti-infective activities of the antimicrobial peptide against intracellular *Mtb* were dependent on the host cell moonlighting glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH) where it functions as a bridging molecule.

P2450/B707

Pseudo-phosphatase Mk-styx Reduces Stress Granules When Autophagy Is Inhibited.

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Pseudo-phosphatase MK-STYX Reduces Stress Granules When Autophagy is Inhibited Tatiana Prioleau and Dr. Shantá D. Hinton the Biology Department, College of William and Mary, Williamsburg, VA, 23185 **ABSTRACT:** Mitogen activated protein kinase phosphoserine/threonine/tyrosine-binding protein (MK-STYX) is a pseudo-phosphatase, which lacks the essential amino acids cysteine and histidine in the signature motif (HCX5R) that ensures proper phosphatase-enzymatic activity. This renders MK-STYX catalytically inactive; however, this does not render the protein useless. Even though it is unable to hydrolyze phosphorylated residues, MK-STYX is still able to bind phosphorylated substrates. Because of

this, MK-STYX as well as other pseudo-phosphatases serve as signals in important signal transduction pathways. We reported that MK-STYX reduces stress granules, the cell's protective response to environmental stressors. Stress granules are cytoplasmic aggregations comprised of stalled mRNA and proteins. They serve as temporary sites of mRNA modification, sorting, and translation of essential proteins during cellular stress. Stress granules have a life cycle that consists of assembly, coalescence, and disassembly. If stress granules persist after the stressors cease, they may become toxic, affecting major cellular pathways such as the cell cycle and protein synthesis. MK-STYX is involved in reducing these stress granules, but it remains unknown how MK-STYX accomplishes this. The aim of this project is to determine whether MK-STYX reduces stress granules through the autophagy pathway; previous research shows that autophagy clears stress granules. Human embryonic kidney (HEK/293) cells were co-transfected with G3BP-GFP (stress granule nucleator) and mCherry or mCherry MK-STYX or mCherry MK-STYX (active) and analyzed with fluorescence microscopy. Cell co-expressing G3BP-GFP and mCherry (control) showed stress granule formation whereas G3BP-GFP and mCherry MK-STYX co-expressing cells showed stress granule reduction, which was expected. To investigate whether MK-STYX clears stress granules through the autophagy pathway, chloroquine, a drug that inhibits autophagy, is used on all transfection conditions, which is juxtaposed with no treatment placed on all transfection conditions. In the presence of chloroquine, HEK/293 cells co-transfected with G3BP-GFP and mCherry MK-STYX showed the reduction of stress granules. This suggests that MK-STYX reduces stress granules independently of the autophagy pathway.

P2451/B708

Involvement of Atg5 in Apoptotic Nuclear Disassembly.

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[Introduction] We have previously reported that nuclei were condensed from stage 0 to 3 in apoptosis by an in *vitro* system using isolated nuclei (Toné *et al. Exp. Cell Res.* 2007, **313**, 3635-3644). Stage 0 means that the nucleus of the cell is in a normal state, while Stage 1, nuclei become a ring. Stage 2 is a state in which the ring of stage 1 becomes a necklace, and stage 3 is a state in which the beads of necklace aggregate interiorly. In addition to Stage 1~3, we found final stage, Stage 4 in apoptosis, in which the beads of necklace aggregates scattered outward. In this study, in order to reveal the role of autophagy, especially ATG5, one of the important factors in autophagy in executing apoptotic nuclear condensation, we examined nuclear condensation would be induced normally in the absence of ATG5. Moreover, we examined the behavior of ATG5 during apoptotic execution after UV irradiation using anti ATG5 antibody. [Methods] Immortalized MEF derived from wild type (WT) or ATG5 knockout (KO) mouse (Kuma *et al. Nature* 2004, **432**, 1032-1036) were irradiated with UVC (100 J/m^2) or treated with 50 μM etoposide, and cultured till appropriate time. [Results] Nuclear condensation of Stage 1 to 4 occurred and many scattered nuclei (nuclear condensation stage 4) were found in WT. But almost ATG5 KO nuclei were not scattered and arrested at Stage 3. We found that ATG5 protein was gathered around the nucleus at early days after UV irradiation by immunofluorescent staining. As for phagocytosis by, WT cells were successfully phagocytosed by macrophage (MΦ), but ATG5 KO cells failed. [Discussion] in conclusion, ATG5 would be involved in transition from stage 3 to stage 4 of nuclear condensation. We also found that ATG5 selectively recognized the nucleus when MEF cells were apoptotic and ATG5-KO was not phagocytosed by MΦ. It can be speculated that the presence of dead cells not phagocytosed by MΦ due to nuclear abnormality would lead to autoimmune diseases. In addition, a significant decrease

in ATG5 has been reported as a risk factor for SLE (Xiao *et al. Int. Immunopharm*. 2016, **40**, 351-361). It would be interesting to know whether cells derived from SLE patients would perform normal apoptosis, especially nuclear condensation.

P2452/B709

Selective Autophagic Clearance of Neurodegeneration-associated Protein Aggregates Is Mediated by the Autophagy Receptor, TAX1BP1.

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Misfolded protein aggregates can disrupt cellular homeostasis and cause toxicity, a hallmark of numerous neurodegenerative diseases. Protein quality control by the ubiquitin proteasome system and autophagy is vital for clearance of aggregates and maintenance of cellular homeostasis. Autophagy receptor proteins bridge the interaction between ubiquitinated proteins and the autophagy machinery, allowing selective elimination of cargo. Aggrephagy, the selective elimination of protein aggregates, is critical to protein quality control, but how aggregates are recognized and targeted for degradation is not well understood. Using CRISPR-mediated gene editing, we examined the requirements for 5 autophagy receptor proteins: OPTN, NBR1, p62, NDP52, and TAX1BP1 in proteotoxic stress-induced aggregate clearance. Endogenous TAX1BP1 is both recruited to and required for the clearance of stress-induced aggregates while overexpression of TAX1BP1 increases aggregate clearance through autophagy. Furthermore, we found that TAX1BP1 is highly and specifically expressed in the brain compared to other autophagy receptor proteins, suggesting a potential protective role in neurodegenerative disease. In addition, TAX1BP1 depletion sensitized IPSC-derived neurons to proteotoxic stress and toxicity due to expression of Huntington's disease-linked polyQ proteins, whereas TAX1BP1 overexpression clears cells of polyQ protein aggregates by autophagy. TAX1BP1 knockout mice exhibited increased accumulation of ubiquitin conjugates in various brain regions compared to wildtype mice, further supporting a role for TAX1BP1 in prevention of aggregate accumulation. We propose a broad role for TAX1BP1 in the clearance of cytotoxic proteins, thus identifying a new factor with therapeutic potential in clearance of protein inclusions.

P2453/B710

Ph.D.

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Cordycepin, the major active component from Cordyceps militaris, has been reported to significantly inhibit some types of cancer; however, its effects on ovarian cancer are still not well understood. In this study, we treated human ovarian cancer cells with different doses of cordycepin and found that it dose-dependently reduced ovarian cancer cell viability, based on Cell counting kit-8 reagent. Immunoblotting showed that cordycepin increased Dickkopf-related protein 1 (Dkk1) levels and inhibited β-catenin signaling. Atg7 knockdown in ovarian cancer cells significantly inhibited cordycepin-induced apoptosis, whereas β-catenin overexpression abolished the effects of cordycepin on cell death and proliferation.

Furthermore, we found that Dkk1 overexpression by transfection downregulated the expression of c-Myc and cyclin D1. siRNA-mediated Dkk1 silencing downregulated the expression of Atg8, beclin, and LC3 and promoted β-catenin translocation from the cytoplasm into the nucleus. These results suggest that cordycepin inhibits ovarian cancer cell growth, possibly through coordinated autophagy and Dkk1/β-catenin signaling. Taken together, our findings provide new insights into the reatment of ovarian cancer using cordycepin.

P2454/B711

Uncovering Active Modulators of Native Macroautophagy through Novel High-content Screens.

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Autophagy is a critical, evolutionarily conserved pathway mediating the breakdown and recycling of cellular proteins and organelles. Emphasizing its pivotal nature, autophagy dysfunction contributes to neurodegenerative conditions, cancer, infectious diseases and cardiac disorders. Development of effective autophagy modulating drugs to treat these diseases has been hampered by fundamental deficiencies in available methods for measuring autophagic activity, or flux. To overcome these limitations, we created a unique human reporter cell line and a non-invasive imaging assay to measure autophagic flux in living cells, without the need for potentially confounding drug treatments or protein overexpression. The photoconvertible protein Dendra2 was introduced into the endogenous *MAP1LC3B* locus of human cell lines via CRISPR/Cas9 genome editing, enabling accurate and sensitive determinations of autophagy activity via optical pulse labeling. High-content screening of 1,200 tool compounds and FDA-approved drugs using this assay showed that many reported autophagy stimulators either fail to enhance autophagy, or conversely inhibit autophagy flux, reaffirming the importance of measuring pathway dynamics rather than the steady state abundance of intermediates. Within an expanded library of 24,000 compounds sampling a wide diversity of chemical space, we identified novel active compounds with profound effects on autophagy, validated by comparison with alternative methods. Further, we demonstrate that NVP-BEZ235, one of the most prominent autophagy activators identified herein, exhibited significant neuroprotective properties in a neuronal model of frontotemporal dementia and amyotrophic lateral sclerosis. These studies thus confirm the utility of the high-content autophagy assay we developed, while simultaneously highlighting new autophagy-modulating compounds that display promising therapeutic effects.

P2455/B712

Autophagic Flux in Oocytes from Pre-pubertal and Peri-ovulatory Rats during Follicular Atresia.

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Autophagy is a regulated and evolutionarily conserved catabolic process that implies the degradation and recycling of cytoplasmatic content (1). Autophagy is present at low levels in normal cells with the aim to maintain the intracellular homeostasis by eliminating damaged organelles and proteins. Identification of diverse proteins involved in the initiation (mTORp and Beclin-1), elongation (Atg8/LC3) and degradation (Lamp1 and p62) can be used to monitor the autophagic flux (2).The follicular atresia is a normal event into mammal's ovary to select the oocyte capable to be fertilized. It has been described the presence of the autophagic cell death process in oocytes of atretic follicles from rats in different ages. Although increased levels of LC3 and Lamp1 in oocytes from rats have been reported (3), the

complete degradation levels of this process have not been studied during oocyte elimination. To explore the autophagic flux, the presence and abundance of pro-autophagic proteins involved in each step of the autophagy were observed in pre-pubertal (19-day old) and peri-ovulatory (28-day old) rats. The results indicate that increased levels of mTORp were presents in oocytes from antral atretic follicles of both stages pre-pubertal and peri-ovulatory oocytes. Differently, mTORp was lower in the secondary and primary follicles. The marker to nucleation of autophagic vesicle Beclin-1 was increased in altered oocytes in all the phases of follicular development, as well as LC3 and Lamp 1. However, the marker to the degradation of the vesicles content p62 was decreased in oocytes from atretic follicles. Our results allowed us to identify that the oocytes from antral atretic follicles are more advanced in the autophagic process, and this is more evident in the oocytes of 19-day old. The increased levels of autophagy-related proteins Beclin-1, LC3, and Lamp 1, and decreased levels of p62 indicate the promoting of autophagy in atretic oocytes. In addition, we further show the activation of autophagic flux during oocyte elimination. These results allow us to conclude that the increased autophagic levels in the atretic oocytes is conducted until the complete degradation of the sequestered cytoplasmic content, indicating that autophagy drives to the oocytes toward an elimination process more than a survival pathway. Acknowledgements: PAPIIT IN227919.(1) Levine and Kroemer, 2008. Cell 132(1):27-42. doi: 10.1016/j.cell.2007.12.018. (2) Seranova E et al., 2019. Methods Mol Biol. doi: 10.1007/978-1-4939-8873-0_26. (3) Escobar ML et al., 2010. Apoptosis. 15(4):511-26. doi: 10.1007/s10495-009-0448-1.

P2456/B713

Mitophagy Receptor Nix Mediates Cd-induced Mitophagy in Absence of Parkin in Hela Cells through Phosphorylation.

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Cadmium (Cd), a non-essential heavy metal, when exposed to cells causes damages to mitochondria. Cell has evolved mechanisms to selectively remove damaged mitochondria known as mitophagy. Just like other heavy metals, Cd induces mitophagy mainly through PINK1/Parkin pathway. However, whether Cd exposure can trigger other mitophagy pathways when PINK1/Parkin is abolished remains elusive. We employed HeLa cells, which lack fully functional Parkin, as a cell model to study Parkin-independent mitophagy pathway induced by Cd. Our results showed that NIX could provide an alternate pathway for Cd-induced mitophagy in HeLa cells where the most potent PINK1/Parkin pathway in heavy metals-induced mitophagy is compromised due to mutations in Parkin gene. Specifically, 10 μM Cd for 12 h induced mitophagy in GM00637 and HeLa cells, which was assessed by mitochondrial fusion to lysosomes and decreased expression of mitochondrial markers such as COX-IV and HSP60. Notably, in GM00637 cells, Cd induced mitophagy was still dominantly mediated by PINK/Parkin pathway as evidenced by translocation of Parkin to mitochondria and co-localization with COX-IV. However, increased expression of NIX was observed in mitochondria of HeLa cells only. Moreover, NIX phosphorylation at serine-81 significantly increased in cells treated with Cd, implying that phosphorylation of NIX plays an important role in NIX-mediated mitophagy. These findings reveal a novel mechanism of Cd toxicity and suggest a compensatory role of NIX in Cd-induced mitophagy.

P2457/B714

Regulation of the Unlipidated LC3/GABARAP Pool in Mammalian Autophagy.

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Macroautophagy is a conserved degradation pathway, featured with the *de novo* formation of double-membrane organelles called autophagosomes (AVs). AV formation requires a critical ubiquitination-like lipidation reaction, that covalently attaches the conserved glycine residue in LC3/GABARAP family proteins to phosphatidylethanolamine (PE) on growing AVs. Like ubiquitin and most ubiquitin-like proteins, LC3/GABARAP family is first translated to produce a pro-form, with an extension of amino acids downstream of the conserved glycine. Normally the extension is cleaved by a cysteine protease family of ATG4 rapidly after translation, to expose the glycine residue to allow for lipidation. However, it is not known why LC3/GABARAP and other ubiquitin-like proteins contain the carboxyl(C)-terminal extension, and thus need to be primed first for conjugation. Here we report novel regulation on the unlipidated, glycine-exposed LC3/GABARAP pool. We found a new LC3/GABARAP form when LC3/GABARAP is expressed with the C-terminal extension removed, thus ending with the conserved glycine residue. We observed similar effects with both LC3B and GL1, and in multiple cell lines. By SDS-PAGE electrophoresis, the new form migrates slightly slower than the endogenous ATG4-primed LC3/GABARAP-I form, or pre-primed recombinant LC3/GABARAP proteins purified from bacteria. In addition, a glycine to alanine mutation in LC3B completely abolishes this new form, strongly suggesting the C-terminus as the modification site. We are currently using mass spectrometry to determine the molecular identity of this new form. In summary, we discovered that non-natually primed LC3/GABARAP with the C-terminal glycine exposed is susceptible to other modifications, suggesting the priming step for ubiquitin and ubiquitin-like proteins may coordinate with other mechanisms to protect the reactive glycine residue at the C-terminus.

P2458/B715

Mutations in Atg11's Coiled-coiled 2 Region Disrupt Binding of Multiple Autophagic Partners.

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Selective autophagy is a cellular process crucial to cellular health and function. This process involves the sequestration and transport of specific cytosolic material such as malfunctioning organelles and protein aggregates to the lysosome / vacuole for degradation and recycling. Patients with perturbed autophagic pathways are often subject to neurodegenerative diseases such as Alzheimer's and dementia. Selective autophagy is mediated by a cascade of specialized proteins referred to as 'autophagy related proteins' (Atg proteins). Many Atg proteins have been shown to interact with Atg11, leading to a model where Atg11 functions as a scaffolding protein and a critical link in the selective autophagic process. These interactions are thought to be mediated through several predicted coil-coil (CC) domains, each of which has a unique set of binding partners. Previous literature has suggested that at least five of these partners, including Atg1, Atg9 and Atg11 itself, bind at Atg11's CC2. The objective of this study was to identify which amino acids within the CC2 are crucial for the binding of each of these partners using directed mutagenesis and a yeast two hybrid screen. We found that mutations at Y565 disrupted binding between Atg11 and each of these partners. However, mutations at Y565 were also found to disrupt binding of Atg11 with Atg32 and Atg19, which are known to bind to the CC4 region of Atg11. This result suggests that mutations at Y565 caused misfolding of the entire Atg11 protein. Consistent with this, western blotting showed that mutations at Y565 caused a significant reduction in Atg11 protein

levels. These results are in contrast to previous reports that removal of the CC2 region does not block interaction with Atg19, and suggest that the previously published mapping of the Atg1, Atg9 and Atg11 interactions to the CC2 may not be reliable.

P2459/B716

Autophagy in Germline Proliferation and Maintaining Genomic Stability.

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Autophagy is a conserved cellular recycling process crucial for homeostasis. In this multistep process, cellular material destined for degradation is enclosed in the autophagosome, a double-membrane bound organelle that fuses with the lysosome for degradation. BECN1 is crucial for the initial nucleation step of autophagosome formation and is a haploinsufficient tumor suppressor in mammals (Liang et al., 1999; Yue et al., 2003; Qu et al., 2003). The role of autophagy in cellular homeostasis is well documented, but its function in stem cell proliferation and genomic stability, remains not well understood. We recently described a role for BEC-1, the *C. elegans* ortholog of mammalian BECN1, and several other autophagy genes in germline stem cell proliferation (Ames et al., 2017). Specifically, we found that BEC-1*,* ATG-18 (in mammals, WIPI1/2), ATG-16.2 (ATG16L) and ATG-7 (ATG7), are required for the late larval expansion of germline stem cell progenitors during development. We demonstrated that BEC-1/BECN1 functions non-cell autonomously to promote germline stem cell proliferation, which may have implications for the understanding and development of therapies against malignant cell growth in the future. A role for BECN1 in the DNA damage response (DDR) to UV, where it exhibits crosstalk with CEP-1, the *C. elegans* ortholog of p53 was also reported (Hoffman et al., 2014). CEP-1 is well known for its roles in promoting genomic integrity through the DDR, including cell cycle control, apoptosis, and DNA repair. CEP-1 was recently shown to act in meiosis, suppressing nonhomologous end-joining (NHEJ), to commit repair of double-strand breaks (DSBs) to the error-free homologous recombination (HR) pathway (Mateo et al., 2016). We are now investigating the mechanism(s) by which BEC-1 and other autophagy genes promote DNA damage repair. Our preliminary data indicate a role for autophagy in promoting meiotic fidelity. Meiotic crossover events are initiated by induction of DSBs, which are faithfully repaired by homologous recombination to maintain genomic stability. The major hallmarks of meiosis include pairing of homologous chromosomes, synapsis, DSB formation, and crossover repair. A defect at any of these steps can result inchromosomal abnormalities and lethality. Our current work seeks to elucidate the steps of meiosis that require autophagy gene activity. To this end, we are examining the role of different autophagy genes, including *bec-1/becn1 and atg-7, in* the promotion of faithful pairing, segregation, crossover formation and resolution of chromosomes in the germline during meiosis.

P2460/B717

Regulation of Atg4 Family Proteases by the C-terminal LIR Motif.

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During macroautophagy in mammalian cells, the LC3 and GABARAP subfamilies of proteins undergo two essential proteolytic cleavage events. The first is a constitutive processing of these proteins from a “pro-

“ form to an activated form-I protein, in which a COOH-terminal glycine is exposed by proteolysis (priming). This glycine is subsequently used in the conjugation of these proteins to lipids on the growing autophagosomal membrane. Later, when the autophagosome is fully formed, these proteins are released from the conjugated lipid by a second ATG4-dependent proteolysis event (delipidation). We recently established that the COOH-terminal LC3-interaction region (LIR) on ATG4B contributes specifically to the deconjugation from lipids but not to proteolysis in solution, suggesting this motif is essential for establishing the temporal release of LC3 from autophagosome membranes. This motif is also conserved in the other three mammalian ATG4 proteins, thus here we test whether delipidation is generally regulated at this motif. We find that although the LIR is needed to maintain wildtype-like activity in both ATG4B and ATG4A, the apparent specificity for delipidation and not priming is limited to ATG4B proteins. In addition, phosphorylatable serines in the immediate vicinity of the LIR only modestly alter the functionality of ATG4B and ATG4A in either fully in vitro or cell-rescue experiments, even as broad manipulation of kinase pathways shifts the form-I/form-II balance. Together, these results suggest that this LIR motif is not a major site of regulation but rather is an integral component of the proteolysis mechanism. We also discover that within our ATG4A rescue experiments there is a very strong suppression of the delipidation activity: GABARAP proteins accumulate in the lipidated form when only ATG4A is available and we do not detect any form-I proteins. This surprising result suggests that removal from the autophagosome either absolutely requires ATG4B in *vivo*, or requires a maturation event that is missing when ATG4B is absent.

Chaperones, Protein Folding, and Quality Control 2

P2461/B718

Hyperthermia Triggers ER Resident Protein Secretion.

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Increased body temperature is often associated with infection and injury; however cancer, blood disorders, and genetic disorders (e.g. malignant hyperthemia) can also result in elevated body temperature. At a cellular level, hyperthemic conditions cause the accumulation of misfolded proteins, trigger endoplasmic reticulum (ER) stress, activate the unfolded protein response, and can ultimately cause cell death. Hyperthermic disruptions to ER homeostasis can affect intrinsic ER functions like protein processing and trafficking, lipid and carbohydrate metabolism, and drug detoxification. The ER also serves as the main intracellular calcium reservoir and, in fact, many ER functions depend on the maintenance of a steep calcium gradient with ER luminal calcium concentrations being much greater than those in the cytoplasm. The connection between hyperthermia, ER calcium, and ER proteostasis remains to be fully elucidated. Our lab previously designed and characterized a *Gaussia* luciferase (GLuc) reporter protein, GLuc-ASARTDL, which contains an ER retention sequence that confers secretion specifically in response to ER calcium depletion. Moreover, the behavior of GLuc-ASARTDL also reflects the behavior of other proteins with ER retention sequences. We demonstrate that increased temperature triggers the redistribution of normally ER resident proteins into the extracellular space. This represents a drug targetable molecular mechanism of ER dysfunction underlying hyperthemic conditions.

P2462/B719

Chemogenomic Profiling Identifies the Hsp70 Co-chaperone Hdj2 as a Hub for Anticancer Drug Resistance.

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Hsp70 is an important molecular chaperone that is elevated in a variety of tumors and regulates the stability of oncoproteins including Ras, Muc1, p53, Cdk2, and Rb. Attempts to develop anti-chaperone drugs for clinical use in the treatment of cancer have been hampered by toxicity issues. Hsp70 is regulated by a suite of co-chaperone molecules that transport unfolded protein "clients" to Hsp70 for folding and stimulate the intrinsic ATPase of Hsp70. Rather than targeting Hsp70 itself, in this study we examine the feasibility of inhibiting of the Hsp70 co-chaperone Hdj2 as a novel anticancer strategy. Bioinformatic analysis revealed that Hdj2 is upregulated and/or mutated in a variety of cancers such as prostate cancer, breast cancer, and stomach cancer. To assess whether inhibition of Hdj2 may sensitize cells to validated anticancer drugs, we screened compounds present in the NIH Approved Oncology collection for chemical-genetic interactions with the loss of Hdj2 in cancer cells. 41 compounds showed strong synergy with the loss of Hdj2, and 3 of these hits are validated in combination with a unique Hdj2 inhibitor (116-9e) for synergy in Castration-resistant prostate cancer cell lines. Interestingly, 18 molecules from the NIH Approved Oncology collection displayed reduced potency in the knockout cell line, 3 of these hits displayed antagonism in combination with 116-9e. Finally, we used a three-dimensional cell culture model, (spheroids) in order to test the efficacy of the drug combination in an environment that replicates the native tumor environment. Taken together, these results suggest that Hdj2 is a hub for anticancer drug resistance and that Hdj2 inhibition may be a potent strategy to sensitize cancer cells to current and future therapeutics.

P2463/B720

Transient Intracellular Acidification Regulates the Core Transcriptional Heat Shock Response.

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The cellular response to heat involves dramatic changes in the regulation of fundamental cellular processes, including the induction of a conserved stress-associated transcriptional program regulated by Heat Shock Factor 1 (Hsf1). Canonically, this activation is thought to be triggered by accumulation of misfolded proteins generated by thermal denaturation. However, temperature increase triggers many physical changes in cells, including transient intracellular acidification which, by unknown mechanisms, is broadly associated with increased stress resistance in eukaryotes. Despite decades of research that have characterized the heat shock response, how and whether acidification influences stress-associated changes in transcription, translation, and growth is largely unknown. Here we demonstrate that intracellular acidification triggered by heat shock in the budding yeast S. cerevisiae is adaptive and promotes rapid activation of Heat Shock Factor 1 (Hsf1). Using multiple methods to measure and control intracellular pH during stress, we demonstrate that acidification universally promotes induction of stress-responsive gene. Furthermore, we have found conditions in which prevention of intracellular acidification suppresses the heat shock response following a physiologically-relevant increase in temperature. Finally, we show that heat-associated acidification specifically promotes the induction of genes controlled by the canonical transcription factor Hsf1, even under conditions where classic models of Hsf1-activation would predict a suppressed response. Our findings reveal a decisive causal role for intracellular pH changes in the eukaryotic transcriptional stress response. In light of our findings, we

consider the possibility that Hsf1 may be activated by non-canonical means that are not the direct result of protein misfolding.

P2464/B721

Role of Hsp70 in Protein Aggregation of Sialic Acid Deficient Cells: Pathological Relevance to Neuromuscular Rare Genetic Disorder.

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Sialic acids are a family of nine carbon acidic amino sugars that are found on terminal position of most glycans structure of glycoproteins. They are involved in stability, turnover and function of glycoproteins primarily affecting immune responses, cell adhesion, cell migration, cell to cell interactions, cell surface receptor stablility, signal transduction, tumor metastasis, wound healing etc. The deficiency of sialic acids in the cell leads to sialic acid metabolism disorders like GNE myopathy (GNEM) and sialuria. Mutations in sialic acid biosynthetic enzyme GNE (UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase) cause neuromuscular rare autosomal recessive disease, GNEM, where muscle weakness slowly progresses the patient towards wheel chair bound state. Although hyposialytion is considered to be the major cause of the disease, recent studies indicate other alternate roles of GNE in cell apoptosis, adhesion, cytoskeletal network and ER stress. No treatment or cure is available that reverse the defect and reduce muscle degeneration, still, some compounds under clinical trials include sialic acid itself and its precursors like N-acetylmannosamine (ManNac), sialyllactose. Pathologically, muscle biopsies of patient reveal occurrence of rimmed vacuole which are protein aggregates of misfolded proteins. In order to understand pathomechanism of protein aggregation in sialic acid deficient cells, HEK cell based model for GNEM was generated where pathologically relevant GNE mutations from Indian subcontinent were over-expressed and stable cell lines were generated. The GNE mutant cell lines were established for hyposialylation, reduced enzymatic activity as well as restoration of phenotype after sialic acid supplementation. The levels of key molecular chaperone, HSP70, was found to be altered in sialic acid deficient cells as observed by immunoblotting and confocal microscopy. p-Tau and β-amyloid proteins showed differential expression in GNE mutant cells compared to wild type control cells. The overall protein aggregation was found to be increased in sialic acid deficient cells compared to control cells using Thioflavin S in FACS analysis. Downstream effect of HSP70 malfunction activates stress kinases, JNK and BAG3 leading to apoptosis. Further, HSP70 function is regulated by cytoskeletal network controlling actin dynamics via cofilin and RhoA. We observed activation of p-cofilin and Rho a in sialic acid deficient cells as compared to control cells. Our study indicate role of HSP70 in protein aggregate formation in sialic acid deficient cells. Further study in GNE knock out muscle cells would validate role of HSP70 in GNE deficient cells. Our study offers HSP70 as potential therapeutic target for drug design for GNEM.

P2465/B722

Gene Knockout of Calnexin Or Calreticulin Could Not Halt Human Prothrombin Secretion.

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Most of secretion proteins are N-glycosylated in lumen of the endoplasmic reticulum (ER). It is generally accepted that lectin chaperone, calnexin (CNX) and calreticulin (CRT) which can bind to a type of N-glycans, promote folding of secretion proteins, and that unfolded proteins are transported back to the cytoplasm, where they are degraded by ubiquitin/proteasome system. Only properly folded proteins are

transported to Golgi apparatus. In order to investigate the quality control of secreted protein mediated by CNX/CRT, we have studied the effects of mutations in secreted proteins and gene disruption of CNX or CRT on their secretion. We chose human prothrombin (FII) as a model of secretion protein and generated all 7 possible mutants on 3 positions of the N-glycosylation motifs on FII and 16 mutants on single amino-acid substitution of prolyl residue. The expression and secretion of the mutants were examined by transient transfection to HEK293 or COS-7 cells. All the mutants in N-glycosylation motifs were secreted into medium, even if the triple mutant which has no N-glycan was secreted, although the amount of secretion was decreased. The secreted mutant FII had the similar specific activity to that of wild FII, after treatment with the snake venom Ecarin. In contrast, two mutants on 137Pro or 376Pro residue were never secreted into medium in the prolyl residue substitution. To examine the effects of CNX or CRT gene disruption on secretion of FII, we established two independent CNX or CRT knockout HEK293 cell lines, respectively. Transfection of wild-type FII into the knockout cells showed efficient secretions, whereas secretion of the single mutation FII, P376L was completely halted in CNX or CRT knockout cells as well as in parent HEK293 cells. Taken together, these facts suggest that lectin chaperone, CNX and CRT could bind directly unfolded proteins without N-glycan. It is noteworthy that ER associated degradation (ERAD) of FII would be unusual. Inhibition of the 26S proteasome by Epoxomicin did not affect the intracellular accumulation of FII in the wild-type and the knockout HEK239 cells. On the other hand, intracellular FII was accumulated by lysosomal suppression with Chloroquine. This suggests that the degradation of unfolded FII may not be mediated by the 26S proteasome but by lysosome.

P2466/B723

Elucidation of Er Stress and Upr Pathway in Sialic Acid Deficient Cells: Pathological Relevance to Gnem.

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UDP-GlcNAc 2-epimerase/ManNAc kinase (*GNE*) is the key enzyme involved in sialic acid biosynthesis. Point mutations in the GNE gene are linked to a rare genetic disorder known as **GNE Myopathy** characterized by slowly progressive distal muscle weakness that begins in the late teens to early adult years. Various studies have shown the role of GNE other than biosynthesis of sialic acid and affecting targets not linking with sialic acid biosynthesis. Pathologically, rimmed vacuole formation due to accumulation of misfolded proteins has been observed in muscle biopsies of patients. In order to understand the phenomenon of protein misfolding in sialic acid deficient cells, a HEK cell based system overexpressing GNE mutations of Indian origin was generated. Ultrastructure study by TEM revealed dilation of ER lumen in GNE mutant cells compared to wild type cells indicative of ER stress. Upregulation of GRP78, ER chaperone, indicated accumulation of misfolded proteins which may elucidate ER stress via UPR pathway through any of the three mechanisms: IRE1, PERK or ATF6 pathway. Activation of IRE-1 branch of UPR in GNE mutant cells was ascertained by increased XBP1 splicing via quantitative real time PCR. PERK and its downstream molecule, eIF2α, showed increased phosphorylation in GNE Indian mutants indicating translational attenuation. Further, ER-redox state as measured by MERO-GFP (Mammalian Endoplasmic reticulum-localized RedOx-sensitive GFP) ratio indicated increased oxidized versus reduced ER state suggesting ER stress in GNE mutant cells. This ER stress may cause activation of UPR pathway leading to cell apoptosis in GNE deficient cells. Interestingly, treatment of GNE deficient cells with cell survival molecules such as IGF-1 reverses the defect due to GNE mutation in the cell. Upon IGF-1 treatment, ER stress is reduced in GNE deficient cells as observed

by XBP1 splicing, PERK/eIF2α phosphorylation and MERO-GFP redox state. IGF-1 could rescue the cell apoptosis of GNE mutant cells, thereby, offering a potential therapeutic target for genetic disorders associated with sialic acid metabolism. Future studies on skeletal muscle cells with GNE knock out would validate the pathomechanism of misfolded protein accumulation and potential drug molecules in GNEM.

P2467/B724

Overexpression of Perilipin-2 Protects Against Endoplasmic Reticulum Stress-mediated Fatty Liver Disease in Zebrafish Hepatocytes.

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Fatty liver disease (FLD) is the most common hepatic pathology worldwide and is characterized by an accumulation of lipid droplets in the hepatocytes. Stress signaling pathways in response to unfolded protein accumulation in the endoplasmic reticulum (ER) are mediated by the unfolded protein response (UPR). This network can be accompanied by morphological changes in ER structure either as a sign of dysfunction or as a mechanism to enhance ER capacity. We investigated the relationship between ER morphological changes, UPR target gene expression and fatty liver using zebrafish expressing a transgenic fluorescent marker of the ER in hepatocytes (*Tg(fabp10a:ER-tdTomato)).* Intravital confocal imaging in 5 days post fertilization (dpf) transgenic zebrafish was performed across 6 genetic and pharmacological interventions that induced ER stress in the liver, including two environmental risks factors for fatty liver (ethanol and arsenic). We developed a quantitative approach to measure ER morphology, and found that in untreated livers, hepatocytes had a static and highly reticular ER whereas all stressors caused extensive loss of reticular ER, with tunicamycin and arsenic causing the most dramatic changes characterized by motile punctae which retained the fluorescent ER label. To assess the correlation between ER structural changes and lipid accumulation, we used a transgenic zebrafish line overexpressing the cytoplasmic lipid droplet protein Perilipin-2 (PLIN2, *Tg(fabp10a:EGFP-hPLIN2)*). Surprisingly, overexpression of PLIN2 blocked ER morphology changes in response to tunicamycin, suggesting that this lipid droplet protein protects against ER stress in the liver.

P2468/B725

Nucleation and Growth of a Phase-separated Huntingtin Inclusion.

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All major neurodegenerative disorders are characterized by the accumulation of large aggregates of unfolded protein in the nervous system. Among neurodegenerative disorders, the etiology of Huntington's Disease (HD) is uniquely simple: the sole cause of HD is a class of dominant mutations in the HTT gene, which lead to the expansion of a polyglutamine repeat region in the huntingtin protein (Htt). Expanded polyglutamine tracts cause instability, unfolding, and aggregation of mutant Htt protein. Although Htt protein is widely expressed in most tissues throughout life, only a subset of cells degenerate; most cells are able to tolerate mutant Htt without significant ill effect. How do cells cope with large quantities of unfolded protein? Many studies have found that the presence of large inclusions of aggregated protein correlates poorly with cell death, and that the concentration of small oligomeric aggregates is more predictive of cell death. Similarly, in the budding yeast *S. cerevisiae*, cells that form a

single ovoid mutant Htt-GFP inclusion grow normally, whereas numerous widely distributed Htt-GFP aggregates are toxic to the cell. Our goal is to elucidate the mechanisms by which inclusions are initiated and material is incorporated into them. We have used quantitative confocal microscopy to show that the mutant Htt-GFP inclusion in yeast is a mobile, gel-like compartment that releases huntingtin protein. Particle tracking of small aggregates indicates that they move randomly, suggesting that the inclusion body (IB) grows through coalescence with small aggregates that diffuse through the cytoplasm. The formation of an IB may be nucleated and occurs even when the ubiquitin-proteasome system has excess capacity. While the Htt-GFP inclusion in *S. cerevisiae* is often viewed as a deposit of insoluble protein which is associated with the vacuole and receives new material through active transport, our high-resolution imaging studies indicate that the Htt-GFP IB is a mobile, gel-like compartment that most likely grows through collision and coalescence with small particles.

P2469/B726

The Ufm-1 Ligase Ufl-1 Regulates the Unfolded Protein Response (upr) and Polyq Aggregation In*C. Elegans*.

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The expansion of polyglutamine (polyQ)-encoding CAG tri-nucleotide repeats in specific genes can lead to age-related neurodegenerative diseases, such as Huntington's disease. PolyQ expansions can result in protein misfolding and aggregation leading to cellular toxicity. However, the molecular mechanisms that regulate polyQ protein aggregation in *vivo* are not well understood. We are using the genetic model *Caenorhabditis elegans to* identify novel genes and mechanisms that regulate polyQ aggregate formation in *vivo and to* analyze their effects on behavior. Expression of YFP-tagged polyQ proteins with different numbers of polyQ repeats in neurons or muscle in *C. elegans* results in the formation of aggregates and cellular dysfunction that increases with age and number of repeats. Here, we tested whether UFL-1, the E3 ligase for Ubiquitin-fold Modifier-1 (UFM-1), affects polyQ aggregates in *C. elegans*. Ufm1 is one of the least well-understood ubiquitin-like proteins. Although the UFM-1 pathwayhas been implicated in erythroid differentiation and protein homeostasis, the specific roles in vivo are not understood. We found that loss-of-function mutations in *ufl-1* results in increased number of polyQ aggregates of *C. elegans* muscle. An imals treated with the proteasome inhibitor Bortezomib also results in an increase in polyQ aggregates, however, this effect is not further enhanced by *ufl-1* mutants, suggesting that the protein folding stress triggered by Bortezomib acts in the same pathway as *ufl-1*. Using a UPR reporter consisting of the BiP/*hsp-4* promoter driving GFP, we found that loss-of-function mutations in *ufl-1* results in increased activation of the UPR. Our results are consistent with a model whereby loss of the UFL-1/UFM-1 pathway causes increased protein folding stress in cells, resulting in increased polyQ aggregation, and activation of the UPR. This study identifies a novel role for the UFL-1/UFM-1 pathway in regulating polyQ aggregation. Future studies will be focused on testing whether other genes in the UFL-1/UFM-1 pathway regulate polyQ aggregate formation, and if the UFL-1/UFM-1 pathway acts cell autonomously in muscle to regulate the UPR and protein aggregation.

P2470/B727

The Role of Calnexin in Regulating ER Proteostasis of RESET Substrates.

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RESET is a protein quality control pathway that clears diverse misfolded GPI-anchored proteins, including human disease mutants of prion protein (PrP) [1], and select transmembrane proteins out of the endoplasmic reticulum (ER) to the Golgi. RESET contrasts with ER associated degradation and autophagy pathways that retain misfolded proteins for degradation at the ER. During RESET, misfolded proteins are released by the ER-resident chaperone, calnexin (CNX), and bound by p24-family members for vesicular transport to the Golgi. The misfolded proteins subsequently transit the cell surface en route to lysosomes where they are destroyed. Thus, steady-state turnover of RESET substrates is concomitant with ER-export. However, physiological and chemical ER stressors dramatically enhance ER-export and consequent lysosomal degradation. Here we address the questions (i) what are the mechanisms regulating constitutive ER-export of RESET substrates during steady-state conditions and (ii) how do ER-stress conditions enhance ER-export of RESET substrates for subsequent degradation? for these studies, we combined imaging with biochemical analysis, including pulse-chase and pull-downs, using a previously characterized misfolding variant of PrP (PrP*[1]) as a model substrate. Our results suggest that the flux of RESET is regulated by competition of other unfolded/misfolded proteins for CNX-binding under steady-state conditions. Chemically blocking new expression of CNX substrates dramatically inhibits steady-state ER-export and degradation of CNX substrates. Conversely, an increase of competitor binding interactions with CNX during physiologically induced ER-stress conditions, such as during the upregulation of specific secretory pathway proteins, increases the rate of ER-export and degradation of RESET substrates. We present a new model that displacement from CNX by newly synthesized CNX substrates regulates the constitutive and stress-enhanced turnover of RESET substrates. [1] Satpute-Krishnan, *et al*. ER stress-induced clearance of misfolded GPI-anchored proteins via the secretory pathway. Cell. 2014; **158**:522-533

P2471/B728

Ufmylation of Rpl26 Links Translocation-associated Quality Control to Endoplasmic Reticulum Protein Homeostasis.

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Protein biogenesis at the endoplasmic reticulum (ER) in eukaryotic cells is monitored by a protein quality control system named ER-associated protein degradation (ERAD). While there has been substantial progress in understanding how ERAD eliminates defective polypeptides generated from erroneous folding, how cells remove nascent chains stalled in the translocon during co-translational protein insertion into the ER is unclear. Here show that ribosome stalling during protein translocation at the ER induces the attachment of UFM1, a ubiquitin-like modifier, to two conserved lysine residues near the COOH-terminus of the 60S ribosomal subunit RPL26 (uL24). Strikingly, RPL26 UFMylation enables the degradation of stalled nascent chains, but unlike ERAD or previously established cytosolic ribosome-associated quality control (RQC), which uses proteasome to degrade their client proteins, ribosome UFMylation promotes the targeting of a translocation-arrested ER protein to lysosomes for degradation. RPL26 UFMylation is upregulated during erythroid differentiation, which helps cells to

cope with increased secretory flow, and compromising UFMylation impairs protein secretion, and ultimately hemoglobin production. We propose that in metazoan, co-translational protein translocation into the ER is safeguarded by a UFMylation-dependent protein quality control mechanism, which when impaired causes anemia in mice and abnormal neuronal development in humans.

P2472/B729

Mechanism of a Memory-enhancing Inhibitor of the Integrated Stress Response.

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The integrated stress response (ISR) is an essential signaling network that enables cells to respond to intrinsic and extrinsic stresses by tuning mRNA translation. The ISR acts through the phosphorylation of the GTPase eIF2, a rate-limiting translation initiation factor. Phosphorylation converts eIF2 from a substrate into an inhibitor of its dedicated guanine nucleotide exchange factor, eIF2B, blocking translation. A drug-like eIF2B activator called ISRIB reverses the effects of eIF2 phosphorylation and restores translation during stress. Remarkably, in rodents, ISRIB enhances cognition and corrects cognitive deficits after brain injury without overt toxicity. To determine its mechanism of action, we solved by cryo-electron microscopy an atomic-resolution structure of ISRIB bound in a deep cleft within a decameric human eIF2B. Formation of fully active, decameric eIF2B holoenzyme depended on the assembly of two identical tetrameric subcomplexes, and ISRIB promoted this step by cross-bridging a central symmetry interface. Structures of eIF2B bound to eIF2 in the phosphorylated and dephosphorylated state revealed eIF2B to be a static platform upon which substrate, inhibitor and drug bind to modulate nucleotide exchange. Based on these structures, an interrogation of the ISR in living cells provides a compelling explanation for both ISRIB's mechanism of activating eIF2B and its lack of toxicity. From this work, eIF2B assembly emerges as a paradigm for translational control and as a promising target for therapeutic intervention for a range of neurological diseases.

P2473/B730

ALS-Linked Mutations in UBQLN2 Alter Stress-Induced Puncta Behavior.

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Neuronal longevity is dependent on effective protein quality control (PQC), and aberrant protein inclusions are pathological hallmarks of many neurodegenerative diseases. Mutations in the proteasome shuttle factor ubiquilin-2 (UBQLN2), which participates in PQC mechanisms in cells, cause some types of familial amyotrophic lateral sclerosis (ALS). The protein also localizes to inclusions in motor neurons of patients with sporadic ALS. We recently showed that UBQLN2 forms puncta with liquid-like characteristics and co-localizes to stress granules (SGs), a type of membraneless organelle whose formation and persistence is hypothesized to precede protein inclusions. However, detailed characterization of UBQLN2 incorporation into SGs is lacking, and the cellular mechanisms impaired by ALS-linked mutations in UBQLN2 remain enigmatic. To address this, we provoked the formation of SGs and UBQLN2 puncta in either HeLa or U2OS cells by inducing oxidative stress with arsenite. We found that arsenite-induced SGs assemble within 10-13 minutes, with UBQLN2 incorporating into these SGs between 25-35 minutes. The late incorporation of UBQLN2 into SGs suggests that the exchange of PQC

proteins between SGs and the cytoplasm plays a role in SG persistence. UBQLN2's participation in stress response appears localization-driven, as increases in expression level were not detected via western blot. Live imaging the response of UBQLN2 with ALS-linked mutations (MT UBQLN2) to arsenite-induced cellular stress demonstrate that these mutations may result in differences in number and timing of formation of phase-separated UBQLN2 bodies compared to wild-type (WT). Collectively, these data suggest a decrease in adaptability of and propensity for puncta formation behavior as vehicles by which mutations in UBQLN2 contribute to ALS pathology. Fluorescence Recovery After Photobleaching (FRAP) is underway to determine differences in protein mobility in both WT and MT UBQLN2 stress-induced puncta. STORM microscopy is being utilized to observe behavior of both UBQLN2 and SG-related protein Ataxin-2 on a molecular level. Our results characterize UBQLN2 incorporation into SGs, and suggest differential stress response behaviors of MT UBQLN2 as a potential disease mechanism. These findings will inform future research that will further elucidate mechanisms by which mutations in UBQLN2 contribute to ALS pathology.

P2474/B731

The Lon Protease's Impact on Bacterial Iron Maintenance.

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Proteases are conserved enzymes that are responsible for removing proteins from the cell. In bacteria, such as Caulobacter Crescentus, the Lon protease is essential for degrading misfolded proteins. It is a part of normal protein quality control. This research involves the use of serial dilution spot assays and microscopy to show how cells lacking the Lon protease are sensitive to hydrogen peroxide, more filamentous, and lack a pink coloration. It has also been observed that varying the concentration of PYE media conditions changes the degree of pink coloration present in the cell. Thus far, the addition of iron to the media has proven to have an inhibitory effect on coloration. A Soret absorbance and emission spectra of the pink color suggests the presence of a heme molecule. Heme is critical for detoxification of oxidative stress and could potentially explain the sensitivity to hydrogen peroxide. Given this, the objective of this research is to discover the origin of the pink phenotype and the Lon protease's role in regulating its expression. We will use a combination of both forward and reverse genetics to identify pathways that influence both oxidative stress and pink coloration. Using this approach, we have isolated mutants that restore pink coloration to our Lon deletion strain. Future work will characterize these mutants in relation to other Lon phenotypes in particular oxidative stress. These studies will enhance our understanding of how the Lon protease influences growth and stress responses in Caulobacter crescentus.

P2475/B732

Propagation of Prions in Yeast by Chimeric *A. Thaliana* Chaperones.

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Prions are highly organized protein aggregates that are known to cause a multitude of neurodegenerative diseases in mammals. Prions are also present in baker's yeast, *Saccharomyces cerevisiae*, but they do not result in the death of the cell. Yeast is a highly useful organism to model prion behavior due the multitude of prions present in the yeast cell and the ability of the prion to be propagated through populations; a phenomenon that is reliant upon the fragmentation of these prions by the chaperone machinery. Sis1, an Hsp40, is specifically essential for prion propagation, but different

prions rely upon distinct parts of Sis1. Recently, we identified six functional orthologs of Sis1 in the model plant *Arabidopsis thaliana* which could replace Sis1 to maintain both cell viability and propagation of strong variants of the prion [*PSI*$^+$]. Here we show that these orthologs vary in their ability to maintain the prion [*RNQ*$^+$] and weak variants of [*PSI*$^+$]. Sequence analyses revealed significant differences in the glycine-rich regions among orthologs. This region of Sis1 has previously been shown to be specifically required for [*RNQ*$^+$] maintenance, and may play some role in the propagation of weak [*PSI*$^+$]. To test this, the glycine-rich regions of two orthologs were swapped, and the chimeric proteins were tested for maintenance of [*RNQ*$^+$] as well as weak and strong [*PSI*$^+$] variants. Preliminary results support the notion that the glycine-rich region dictates the ability of variants to propagate [*RNQ*$^+$] but not weak [*PSI*$^+$]. Additional experimentation should provide useful information on the specific parts of Sis1 needed for weak [*PSI*$^+$] propagation in addition to a better understanding of interactions between Sis1 and yeast prions.

P2476/B733

Analyzing Amino Acid Content of Yeast Prion-forming Domains and Its Effect on Hsp104-mediated Prion Curing.

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Yeast prions are self-propagating protein aggregates that act as non-Mendelian genetic factors. In *Saccharomyces cerevisiae,* at least 12 proteins are known to form prions. We have previously noted significant correlations among the amino acid content of the prion-forming domains (PrDs), chaperone sensitivities, and formation rates of the yeast prions [*URE3*], [*SWI*$^+$], [*RNQ*$^+$], [*PSI*$^+$], [*MOT3*], and [*ISP*$^+$]. Among these prions, N-rich PrDs correlated with high levels of specific aliphatic hydrophobic residues and low amounts of glycine and tyrosine. Those prions were also the most sensitive to alterations in chaperone activity. Thus, we hypothesized that prions with N-rich PrDs are less stable in the population than Q-rich prions because their aggregates are less frangible, and likewise that these prions should appear more frequently. Alberti and colleagues in the Lindquist group used an algorithm to identify additional potential prion-forming proteins in yeast; 21 of their most successful candidates together with Sfp1 were later defined by the Ross group as the “Alberti dataset”. Here we present a statistical analysis testing a new hypothesis, based on recent work by Ross and colleagues, that N-rich PrDs are more able to house strongly amyloidogenic, aliphatic hydrophobic residues, whereas Q-rich PrDs will contain more aromatic residues, particularly tyrosine. The relationship between Q/N content, percentage of specific aliphatic hydrophobic residues, and percentage of aromatic residues in the PrDs of these six yeast prions and in the full Alberti dataset were examined. Within the set of six prions previously considered, a strong positive correlation was found between Q/N ratio and %(Y+F+W) and a negative correlation was found between Q/N ratio and %(I+L+M+V), though these correlations disappeared in the full Alberti dataset. Additionally, the effect of Hsp104 overexpression on some prions of the Alberti dataset has been tested with the long-term goal of analyzing the potential effect of amino acid content on this chaperone-prion interaction. These experiments also test the hypothesis that the M-domain of Sup35 is absolutely necessary. These prions are being analyzed using [cPrD-Sup35C] chimeric prions from the Lindquist Lab to ensure only the PrD is causing changes in Hsp104 interaction. Unfortunately, most aggregates were undetectable using SDD-AGE due to the instability of the aggregates, likely due to the lack of the Sup35 M-domain. Successful gels identified no Hsp104-mediated curing of chimeras [Asm4-Sup35C], [Nrp1-Sup35C] or [Gln3-Sup35C], supporting the hypothesis that the

M-domain of Sup35 is responsible for the specific genetic interaction between Hsp104 overexpression and the [*PSI*$^{+}$] prion.

P2477/B734

Hsp70 Independent Role of Nucleotide Exchange Factor Fes1 in Degradation of Gluconeogenic Enzyme and Cell Wall Integrity.

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Cellular protein homeostasis is maintained by regulated protein biosynthesis and degradation of proteins by a protein quality control system (PQC). The PQC is composed of Hsp70, Hsp90 and other co-chaperones (Fes1). The PQC facilitates protein folding, prevents protein aggregation, helps to refold misfolded proteins or stimulates degradation of terminally misfolded proteins. Fes1 is one of the three cytosolic nucleotide exchange factor (NEF) in yeast that requires for growth at high temperature, prion propagation, repression of the stress response and proteasomal protein degradation in association with Hsp70. All known functions of Fes1 are thought to require its ability to regulate Hsp70. The Hsp70 Ssa2, but not its paralog Ssa1, is essential for vacuole import and degradation (Vid) of gluconeogenic enzymes including Fbp1. Fbp1 follows two different degradation pathways depending on the length of glucose starvation. In order to determine if Ssa1 and Ssa2 are regulated differentially by nucleotide exchange factors in yeast, here we find only Fes1 is essential for vacuole import and degradation (Vid) of gluconeogenic enzyme Fbp1. Interestingly differences in Fes1 interaction with Ssa1 and Ssa2 did not account for Ssa2 specificity in the vid pathway. We also find Fes1 mutant (Fes1A79R/R195A) that did not interact with or regulate Hsp70 still retained activities that allowed support of the Vid pathway. Fes1 bound to Vid substrate Fbp1 in vitro independent of Hsp70. Our genetic approaches allowed us to link *fes1Δ* cells thermo-sensitivity with cell wall defects. We also found that Fes1A79R/R195A mutant defective in Hsp70 interaction support growth at high temperature in agreement with this finding, we observed increased phosphorylation of cell wall integrity (CWI) protein Slt2 even at the permissive temperature indicating Fes1 is linked to the CWI pathway. Our study has found the interaction of Fes1 with CWI protein Slt2, increase the possibilities of nucleotide exchange factor Fes1 to work in other cellular events way beyond as cochaperone for Hsp70. These unexpected findings challenge current assumptions in the field about this class of Hsp70 regulatory factors.

P2478/B735

Organelle-targeted Small Heat Shock Proteins in Growth and Stress Tolerance.

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Molecular chaperones are proteins found in all kingdoms of life and are essential to cell survival. Small heat shock proteins (sHSPs) are one class of ubiquitous molecular chaperones that are upregulated during heat stress. They are proposed to act as the first line of defense by binding to heat-sensitive proteins and preventing their irreversible aggregation. Many details of sHSP function remain to be discovered, and exactly what proteins they protect is unresolved. In addition to the cytosolic sHSPs found in other organisms, during heat stress higher plants produce nuclear-encoded sHSPs targeted to chloroplasts and mitochondria, organelles essential to energy metabolism. In *Arabidopsis thaliana* the four sHSPs found in these organelles are: HSP23.5-M/C and HSP23.6-M/C, which are proposed to be dual-targeted to both organelles, HSP25.3-P, which is targeted to the plastids only, and HSP26.5-MII, which is specific to mitochondria. We have found that plants carrying single, double, triple, or quadruple

mutants of these sHSPs appear to grow normally in the absence of stress. Assays of mutant heat sensitivity indicate that the triple mutant of HSP23.5-M/C, HSP23.6-M/C, and HSP26.5-MII shows heat-sensitivity, though the mutant is more tolerant than a mutant of HSP101, the Arabidopsis homologue of yeast chaperone HSP104. The mutants are being tested for other stress phenotypes, and confirmation of the dual-localization of HSP23.5-M/C and HSP23.6-M/C is in progress. Understanding the phenotypes of these sHSP mutants and determining what proteins they protect will bring us closer to defining their mechanism of action, and the mutants will provide a platform for further studies of sHSP structure and function.

P2479/B736

Bax Inhibitor-1 (bi-1) Deficient Mice Develop Hepatic Steatosis by Modulating Aberrant Er Stress during the Aging Process.

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Aging-associated ER stress occurs due to declined chaperoning systems of the ER. Persistent stress in the ER reduces cellular functions, induces apoptosis, and leads to age-related diseases. The main objective of our study was to investigate the effect of BI-1 on aging-associated fatty liver. We categorized wild-type (WT) and BI-1 knockout (KO) mice into young, middle and old groups, and observed the effect on hepatic lipid accumulation in normal food diet condition. We observed that the BI-1 deficient aged mice gained body, and liver weight, and high hepatic triglyceride level when compared with its WT counterparts. The increase in lipid peroxidation level and ROS-induced oxidative stress was observed in BI-1 KO aged liver. Loss of BI-1 induced excessive ER-mitochondria interactions and high molecular weight complexes of protein disulfide isomerase (PDI) during the aging process. Furthermore, the impaired UPR function was observed in BI-1 KO mice in advanced age. The canonical UPR arms proteins were aberrantly expressed where we observed that the expression of ER chaperone proteins like GRP78 and PDI; ATF6 alpha, p-PERK, and p-eIF2 alpha were downregulated and IRE1-mediated JNK signaling was activated in BI-1 KO aged liver. CHOP was highly expressed and the nuclear level of sXBP1 was suppressed in BI-1 KO aged mice. ER-associated degradation was highly impaired in BI-1 deficient aging mice as compared to its comparable group. In addition to TUNEL positive cells, caspase 7 and caspase 12 were highly activated in BI-1 KO mice from the middle to advanced age, suggesting the protective effect of BI-1 on aging-induced apoptosis. Collectively, these results suggest that aging-induced fatty liver is regulated by BI-1 through the regulation of ER stress and proteostasis. **Keywords:** BI-1; aging liver; steatosis; ER stress, chaperones; apoptosis

P2480/B737

Investigating the Ribosome-associated Regulation of Stress Induced Prion Formation and the Physiological Impact of [PSI+] in S. *cerevisiae*.

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In yeast, the [*PSI*$^+$] prion results from a self-propagating amyloid form of the translation termination factor Sup35 and results in elevated levels of nonsense suppression. The rate of *de novo* Sup35 conversion into the prion conformation is known to increase in response to environmental stressors and can produce beneficial phenotypes for [*PSI*$^+$] strains. Thus, the presence of the prion may serve as an

epigenetic switch to allow the heritable propagation of [*PSI*$^+$] acquired changes. Understanding the mechanisms that control prion formation and the resulting physiological consequences is a critical area of research towards furthering the understanding of amyloid biology. Previous work by our lab and others has shown that the ribosome-associated complex (RAC) suppresses prion formation in yeast. The Hsp40 RAC chaperone Zuo1 anchors the RAC to the ribosome and stimulates the ATPase activity of the Hsp70 chaperone Ssb. Unfolding of the C-terminal domain of Zuo1 facilitates its dissociation from ribosomes, where it can activate a transcription factor for the pleiotropic drug resistance (PDR) pathway. Thus, through this mechanism, some environmental stress conditions may decrease the occupancy of RAC on ribosomes, thereby increasing prion formation. In order to study the mechanisms by which cells regulate prion formation in response to environmental stressors, we conducted a large-scale screen looking for factors that promote Zuo1 dependent activation of PDR. We have identified Btt1, a component of the nascent chain associated complex NAC, as a mediator of Zuo1 dependent PDR activation. The increase in the acquisition of prion conformations under stress conditions has been shown to confer in many cases an increased resistance to those environmental stressors. In order to investigate the observed difference in survival, we previously used SILAC (Stable Isotope Labeling by Amino Acids in Cell Culture) to identify changes in the proteome between [*PSI*$^+$] and *[psi*$^-$] isogenic strains. One group of proteins showing marked prion-dependent differences in abundance are members of the Hxt family of glucose transporters. Thus, we are currently examining the physiological consequences of the [*PSI*$^+$] prion on yeast metabolism by utilizing glucose uptake assays and growth competition assays to measure the functional differences between isogenic [*PSI*$^+$] and [*psi*$^-$] strains exposed to a variety of growth conditions.

P2481/B738

Quantitative Proteomics Identifies $GABA_A$ Receptors Proteostasis Network Components.

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GABA type a receptors bind GABA and inhibit neuronal firing. Maintenance of $GABA_A$ receptor protein homeostasis (proteostasis) in the cell utilizing its interacting proteins is essential for the function of $GABA_A$ receptors. How the proteostasis network orchestrates $GABA_A$ receptor biogenesis in the endoplasmic reticulum (ER) is largely unexplored. To address this systematically, we employed a proteomics-based approach to identify the interactomes of $GABA_A$ receptors in HEK293T cells by carrying out a quantitative immunoprecipitation-tandem mass spectrometry (MS) analysis by stable isotope labeling by amino acids in cell culture (SILAC). To enhance the coverage and reliability of the identified proteins, we performed comparative proteomics by using both wild type (WT) α1 subunit and a well-characterized misfolding-prone α1 subunit carrying the A322D mutation as the bait proteins. The WT α1 interactome contains 114 proteins, the α1(A322D) subunit interactome contains 103 proteins, and 52 proteins overlap within two interactomes. Bioinformatics analysis enabled us to identify chaperones, folding enzymes, and ER-associated degradation (ERAD) factors. We identified major chaperone networks, including Hsp70/Hsp40 systems both in the ER (BiP and ERdj3) and in the cytosol (Hsc70, DNAJA1 and DNAJA2), Hsp90 systems both in the ER (Grp94) and in the cytosol (Hsp90β, AHSA1, and UNC45A), N-glycoprotein folding systems (calnexin and UGGT1), Hsp47, and folding enzymes (ERp57 and PDIA1). In addition, we identified ERAD factors, including a number of ubiquitin E3 ligases (HUWE1, UBR5, LTN1, and TRIM21), and retrotranslocation proteins (SEL1L and VCP). To evaluate whether such an interaction network is utilized in the mammalian central nervous systems, we used mouse brain homogenates to carry out co-immunoprecipitation assay. Indeed, we demonstrated endogenous

interactions between α1 and selected WT α1 interactors as mentioned above. Manipulating the GABAA receptor proteostasis network components is a promising strategy to enhance GABAA receptor function to ameliorate related neurological diseases.

Computational Cell Biology/Bioinformatics

P2482/B740

Non-uniform Distribution of Myosin-mediated Forces Governs Red Blood Cell Curvature through Tension Modulation.

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The biconcave disk shape of the mammalian red blood cell (RBC) is unique to the RBC and is vital for its circulatory function. Recent experiments have demonstrated that the biconcave shape of the RBC relies not only on the physical properties of the membrane but also depends on the molecular constituents of the membrane cytoskeleton, including the contractile activity of the nonmuscle myosin IIA (NMIIA) motor protein. Here, we use the classical Helfrich model for the RBC membrane and incorporate heterogeneous force distributions along the membrane to mimic the contractile activity of NMIIA. We find that the biconcave shape of the RBC depends on the ratio of forces per unit volume in the dimple and donut regions of the RBC. Experimental measurements of NMIIA densities at the dimple and donut validate our prediction that (a) membrane forces must be non-uniform along the RBC membrane and (b) the force density must be larger in the dimple region than the donut region to produce the observed membrane curvatures. Furthermore, we find that the tension of the RBC membrane plays an important role in regulating this force-shape landscape. Our findings of heterogeneous force distributions on the plasma membrane for RBC shape maintenance have implications for shape maintenance of many cell types.

P2483/B741

Machine Learning Predicts States of Aging and Disease Based on Emergent Single-cell Mechanical Phenotypes.

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The mechanical properties of cells are continuously subject to change in the context of differentiation, chronological age, and malignant transformation. Thus, our work is poised to demonstrate that these mechanical properties can be exploited and may present novel label-free biomarkers of clinically relevant functional cell states. We have implemented the use of a novel mechano-node-pore sensing (mechano-NPS) method, which is a powerful tool in multi-parametric single-cell analysis that simultaneously measures cell diameter, resistance to compressive deformation, transverse deformation under constant strain, and recovery time after deformation. Based on these measurements we have defined a new dimensionless parameter, the whole-cell deformability index (wCDI), which can be utilized to discriminate between different cell types. Mechano-NPS measurements were shown to be remarkably sensitive to pharmacologically induced alterations of the cytoskeleton. We are now utilizing machine learning algorithms to identify distinguishing mechanobiological features of different cell types in a non-biased manner. Indeed, using this approach we are now generating a database of the

mechanical phenotypes of normal human mammary epithelial cells (HMEC) derived from primary tissue from women age 16 to 91, whose cells are in a range of states from normal to malignant, and with a number of defined signaling pathway alterations. Our machine learning model predicts the chronological age of tested HMECs with high accuracy and can predict pathways that differ between cell types based on patterns of mechanical measurements. Further, we are investigating the molecular underpinnings of the age dependent mechanical phenotypes. Knockdown experiments of the intermediate filament of keratin 14 (KRT14) partially restored a young mechanical phenotype in HMECs isolated from post-menopausal women. Thus, suggesting a role for KRT14 and the cytoskeletal network in cellular aging. Mechano-NPS has effectively identified mechanical phenotypes that can be exploited to detect and predict cellular properties and its potentially clinically-relevant implications in bias-free disease detection are powerful.

P2484/B742

Active Matter Self-organization Simulator (AMSOS): Combining Biophysics and Mechanics on HPC.

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We have developed a simulation package to study the large-scale behavior of biofilament/motor-protein assemblies. Each motor-protein is explicitly tracked in space and its interactions with filaments are described by a two-stage explicit kinetic Monte Carlo model. Binding-unbinding events of a motor are based on free-energy changes between unbound, singly-bound, and doubly-bound states, in a way that preserves detailed balance. Motor-protein binding two filaments walk and diffuse along the filaments thus moving the filaments relative to each other. We use a new numerical method based on geometrically constrained optimization to guarantee that steric interactions between filaments are properly and efficiently handled. We further extended the virial stress formula from classical point-mass systems to rigid bodies with finite volumes. These developments are integrated into a massively parallel software package - AMSOS -- whose application we demonstrate by a self-contracting microtubule-motor assembly of more than 100,000 microtubules.

P2485/B743

Decoding the Variance in Intracellular Organization of the Undifferentiated hiPS Cell.

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The Allen Institute for Cell Science is developing a state space of structural signatures of the undifferentiated human induced pluripotent stem cell (hiPSC) to understand the principles by which cells organize and transition between states (cellular morphogenesis). To do this we take advantage of the ~35 endogenous fluorescently tagged hiPSC lines in the Allen Cell Collection (www.allencell.org), each expressing a monoallelic EGFP-tagged protein representing a particular organelle or structure. We develop image-based assays and segmentation algorithms for quantitative analyses, taking advantage of thousands of replicate high resolution 3D images for each structure. We are investigating biological sources of cellular variation to identify the basis functions of a dimensionally-reduced, interpretable parameter space that represents integrated intracellular organization. We prototyped an analysis workflow based on the nucleus, using lamin B1, as our first key cellular structure. We used the Allen Cell Structure Segmenter to create accurate 3D segmentations of the nuclei in multi-hour 3D timelapse movies. We fit these extracted nuclear shapes using spherical harmonic functions. We analyzed the

contributions of the spherical harmonic coefficients to the variations in nuclear shapes and found that nuclear shape could be well described by five coefficients representing three distinct shape modes. Each nuclear shape mode represented a different source of biological variation in hiPS cell colonies and occurred on a distinct timescale. The mode represented nuclear volume, which increases throughout interphase, occurring over a one day timescale. The second mode represented how flat (vs. round) a nucleus appeared in the apical-basal axis (Z-direction). This nuclear 'flatness' was linked to differences in cell packing in distinct regions of the colony, i.e., more densely packed regions were composed of taller cells with rounder nuclei, when compared to the less densely packed, shorter cells with flatter nuclei that were often found in the center of colonies. Individual nuclei exhibited little variation in flatness over a multi-hour time period, consistent with the longer timescale of cell packing within colonies that occurs over several days. The third mode represented how 'squeezed' a nucleus appeared in the XY plane; this arose from constant interactions between neighboring cells occurring at timescales of minutes or less. We are now applying these analyses to develop biophysical models of nuclear shape and colony dynamics. This general analysis framework will be extended to cell shape and to each of the key intracellular structures in an integrative fashion.

P2486/B744

Mathematical Modelling of Gliding Motility and Its Regulation in *Myxococcus Xanthus*.

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Myxococcs xanthus manages "social" behaviors, such as cooperative feeding and fruiting-body formation, through intercellular communication and coordination. A particular intriguing intercellular coordination lies in coordination of motility between cells with physical contacts. Specifically, *M. xanthus* cells glide on substrate with periodic reversals, and physical contacts with opposite-moving colony mates regulate the reversal frequency. This contact-dependent motility coordination is known to be necessary for intriguing population patterns, such as rippling waves. Frz proteins, regulators that control the periodic reversals in *M. xanthus*, exhibit subcellular dynamics that are highly correlated with that of the gliding motility motors and change in response to cell-substrate and cell-cell contacts. To understand how control and coordination of *M. xanthus* motility works, we developed a mathematical model to capture the coupling between the Frz regulators and the gliding motility machinery in cell reversal control. Using the model, we identified several possible mechanisms through which cell-cell contacts can induce proper responses in cell reversals required for generating the rippling waves. Interestingly, we found that both phase advance and phase delay in the cell reversal upon cell-cell contact were able to generate rippling waves. Our model has proposed a new mechanistic explanation for contact-dependent motility coordination in *M. xanthus.*

P2487/B745

Substrate Selectivity of DNA Dependent DNA Polymerases Is Controlled by Free Energy Barriers.

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The interaction of regular dNTPs with their natural cell targets DNA Dependent DNA polymerases (DNA pols) is multistage process. It includes primary recognition dNTP and DNA-bounded enzyme, nucleotide incorporation into active site. The next milestone is close complex or excluding dNTP from active site.

The excluding of dNTP can be mentioned by structural inconsistency. Furthermore, the existing kinetic models do not completely explain the process of transition from open to close enzyme form after binding of non-complementary dNTP. Thus, it seems to be necessary evaluate the changes of free energy during dNTP migration from cytoplasm/karyoplasm to DNA pol active site. For studying we applied the umbrella sampling which accurately describes the states of molecular system and the free energy changes alone reaction coordinate. Free energy profiles were calculated for ternary complexes of *E. coli* DNA Poll II and human DNA Pols β and λ (high fidelity B and middle fidelity X DNA pol families). These complexes contained one of four regular dNTP or oxidized 8-oxo-dGTP. Matrix nucleotides in DNA pol active center also were differ. The umbrella sampling calculations were performed with GROMACS software using Charmm27 force field and 1320 trajectories in 30 ns each were computed. The weighted histogram analysis method was used for PMF extraction and free energy calculation. It was established, that in most cases (in exception some cases with incoming dTTP) for fittinng of the incoming nucleotide into the polymerase active center it's necessary to overcome free energy barrier, the value of which varies from 2 to 10 kcal /mol. The magnitude of the barrier depends on both incoming and matrix nucleotides. In the case of complementary incoming and matrix nucleotide the energy barrier is not more than 2.5 kcal/mol and it can be overcome due to the energy of thermal fluctuations. In opposite, the non-complimentary binding demands at least 4 kcal/mol of additional energy and forming of such complex is thermodynamically improbable. In the case with incoming 8-oxo-dGTP, the least free energy barrier is shown for its pairing with the matrix dA, that is fully consistent with experimental data. Thus, the difference of free energy barriers required for dNTP fitting into DNA pols active sites seems to be one of the **key factors** controlling the level of their mismatched incorporation in grow DNA chain.

P2488/B746

Propagation of Forces in Epithelial Monolayer Is Highly Dependent on Substrate Stiffness.

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Mechanics and physical forces influence many epithelial processes, including epithelial formation and migration, as well as cell signaling via a process known as mechanotransduction. Many pathological conditions lead to changes in the epithelial mechanical homeostasis. For example, in tumors both the cells and the extracellular matrix become stiffer. Unfortunately, it is not well understood how these mechanical changes affect the signaling between cells. To study this, we used a computational approach to see how mechanical disturbances in the epithelia spread and how the stiffness of the substrate under the cells affects the force propagation. Our cellular modeling approach uses a so-called boundary-based method, where the cells are represented by closed boundary polygons and the various cellular cytoskeletal components and processes are described by springs between or forces affecting the polygon vertices. The substrate is modeled as a mass-spring model, described by a hexagonal spring network. We mechanically disturbed the system by moving one cell to describe local micromanipulation of the epithelial monolayer. We simulated the micromanipulation for the epithelium-substrate system as well as for only the substrate. We found that there is a dramatic difference on how the cell deformation spreads in the epithelia and in the substrate depending on the substrate stiffness. When the substrate is softer than the cells, the deformations caused by micromanipulation of a cell propagate across all the cells in the simulated area and the underlying substrate. However, with stiffer substrate, the deformations in both the other cells and substrate are local; only a few nearest neighbor layers deform and there is very little deformation in the substrate itself. We also simulated the deformation of only the substrate without the cells with similar micromanipulation and found that deformations in the

soft substrate are local whereas the stiff substrate has more global deformations. Our results indicate that the distance traveled by the mechanical deformations, and thus mechanical forces and signals, is highly dependent on the stiffness of the cell substrate. There is a major change in force propagation behavior as the substrate becomes stiffer than the epithelial monolayer, which may be extremely relevant especially in tumors due to the changes in mechanical properties.

P2489/B747

Mathematical Modeling of Centrosome Clustering.

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Centrosome clustering is strongly implicated in cancer cell mitosis. Most tumor cells have extra centrosomes, which would be expected to induce multipolar cell division and consequently fatal, massive chromosome aberrations in daughter cells. However, cancer cells often evade this fatal outcome through clustering the centrosomes and undergoing pseudo-bipolar divisions. Centrosome clustering is mediated by mechanical interactions between microtubules and microtubule-associated motors. To understand the process and necessary conditions for centrosome clustering, we built a model which characterizes the mechanical interactions between centrosomes in a simplistic manner. Our result shows that attractive interaction between centrosomes alone leads to either nearly no clustering or a high frequency of monopolar clustering. After including a repulsive interaction between centrosomes in the model, we are able to avoid monopolar clustering and reproduce the experimentally observed frequencies of bipolar, tripolar and tetrapolar divisions in cells with four centrosomes. This result indicates the importance of forces that push centrosomes away from each other, which likely stems from anti-parallel alignment and sliding between microtubules associated with different centrosomes.

P2490/B748

Modeling Growth-mediated Motility in *Clostridium Perfringens*.

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Many bacteria species are able to expedite colony expansion through motility of the cells. *Clostridium perfringens*, the primary cause of lethal gas gangrene, exhibit a unique mode of colony expansion. Chains of cells continuously grow outward from the bacterial colony and curve. These bacteria appear to lack a direct motility mechanism in individual cells and are hypothesized to rely on bacterial growth to push adjacent cells in the strongly connected cell chains. Interestingly, these cell chains tend to curve as they grow. Using a “rigid-rod” model we simulate the growth dynamics of these bacteria chains. Our preliminary results suggest that the cell chain curvature cannot result from the growth of the cell chain and its interaction with the substrate. Motivated by the observation that multiple chains growing side-by-side appear to curve more than single chains, we hypothesize that chain curvature may be a result of lateral interactions between cell chains. An expanded version of the rigid-rod model is used to include these lateral interactions and also implements collision dynamics between cells in adjacent chains. Ultimately, we will use these mathematical models to investigate if this expansion mode of *C. perfringens* could be advantageous for spreading and surviving on different substrates and environments the bacteria may encounter during their opportunistic life cycle.

P2491/B749

Robust and Automated Detection of Subcellular Morphological Motifs in 3D Microscopy Images.

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Rapid developments in live-cell 3D microscopy enable imaging of cell morphology and signaling with unprecedented detail. However, tools to systematically measure and visualize the intricate relationships between intracellular signaling, cytoskeletal organization, and downstream cell morphological outputs do not exist in 3D. Here we introduce u-shape3D, a computer graphics and machine learning pipeline to probe molecular mechanisms underlying 3D cell morphogenesis and to test the intriguing possibility that morphogenesis itself affects intracellular signaling. u-shape3D includes a generic morphological motif detector that automatically finds 3D subcellular surface structures. It does so by decomposing the cell surface into convex patches, optionally merging these patches, and then classifying them by motif. Merging and classification is performed via support vector machines, a machine learning algorithm. Training data is generated in part by presenting users with the segmented cell surface and asking users to click on examples of motifs. We demonstrate the robustness of this motif detector by applying it to diverse systems, including blebs on melanoma and U2OS cells, filopodia on bronchial epithelial and melanoma cells, lamellipodia on dendritic and T cells, microvilli on breast cancer cells, extensions on microglial cells, and spines on neurons. These cells were imaged via various imaging modalities including high-resolution light-sheet microscopes, such as the lattice light-sheet, a standard commercial light-sheet microscope, and a laser scanning confocal microscope. In addition to motif detection, we also provide 3D tools to measure molecular localization near the cell surface, surface motion, and spatial statistics. All of these tools, as well as the motif detector, are accessible via a graphical user interface. As an application, we measure the differential association of PIP_2 and $Kras^{V12}$ with blebs on melanoma cells. Both signals polarize with blebs and associate with bleb edges, as expected for membrane-localized proteins, but only PIP_2 is enhanced on blebs. That $Kras^{V12}$ and PIP_2 spatially segregate with blebs, suggests that blebs may modulate intracellular signaling. Overall, our computational workflow enables the objective, automated analysis of the 3D coupling of morphodynamics with cytoskeletal dynamics and intracellular signaling.

P2492/B750

Quantifying Cell Biology: Beyond Human Vision.

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Microscopy images contain tremendous information about the state of cells, tissues, and organisms. Often, biomedical researchers use software to identify biological structures and extract metrics of interest from cell images; they quantify phenomena in conventional microscopy experiments as well as high-throughput screens to test drugs in disease model systems. We have developed computational approaches and open-source software, including CellProfiler, for this, bridging the gap between biologists' needs and the latest computational science. Here, we describe going beyond human vision and biologists' existing hypotheses in a strategy called image-based profiling. Instead of measuring individual phenotypes that biologists already know are relevant to a particular disease, we stain many cellular components and extract thousands of morphological features from each cell's image, often using an assay called Cell Painting and computational approaches such as deep learning. We then

harvest similarities in these “profiles”, analogous to transcriptional profiling but less expensive and, surprisingly, more information-rich. Through this approach, we discover that unlabeled cells can be classified for their cell cycle and mitotic stage without any DNA labeling. We find that leukemia can be detected in unlabeled cells, eliminating the need for specific biomarker-based reagents. We find that degradation of red blood cells during blood bank storage can be detected, eliminating the need for tedious expert counting. We find that similarities in images of cells that overexpress genes contain sufficient information to determine unstudied genes' pathway identity and relationships. We identify cell biological impacts of rare disease alleles and mental illnesses. We find that cancer-associated alleles can be classified based on their functional impact without creating individual functional assays for each gene. Overall, cell images should now be considered a source of powerful data about cell state.

P2493/B751

On the Transmission of Spatial Information Via a Physicochemical Channel.

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Environmental stimuli are non-uniformly distributed across space. Living things must represent these distributions in order to use this spatial information about the stimuli to compute an appropriate behavioral response. Biological systems often represent and transmit information through chemical channels where information is represented using diffusible molecules. However, spatial information is challenging to represent and transmit through chemical channels because diffusion promotes spatial information loss. In this work, we introduce an information theoretic framework to identify fundamental limits associated with transmitting spatial information in biological reaction-diffusion systems. To analyze the representation and transmission of spatial information by cellular signal transduction pathways, we construct a minimal reaction-diffusion channel capable of transmitting spatial information across a cell membrane. Our channel model consists of a cell membrane and a membrane-bound, diffusible response molecule. The membrane separates the cytosol from the environment. Given inducer molecules in the environment, our physical channel allows for the spatial distribution of the inducer to be transmitted to the cytosol via the arrangement of response molecules on the inner membrane surface. From this simplified model, we obtain a channel transition function that maps the inducer distribution (input) to a set of possible response molecule arrangement on the membrane, with their associated probabilities (output). The transition function allows us to establish bounds on channel performance, and characterize trade-offs between the rate and accuracy of spatial information transfer, for different signal transduction architectures. We show that receptor clustering is important for information transmission in temporally stable environments, whereas uniform distributions of receptors are more beneficial in highly dynamic environments. In conclusion, we establish a theoretical framework for studying trade-offs of spatial information transmission via a biochemical communication channel. Our results offer insights into the functions of natural biological channels such as transmembrane signal transduction in immune sensing, and the designs of novel nanosensors.

P2494/B752

Emergence of Multicellular Calcium Synchronization by Information-transfer between Individual Cells.

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We combined a unique experimental system with a data-driven modeling approach as a platform to quantitatively evaluate how noisy heterogeneous behavior of individual cells is integrated across a population toward multicellular calcium synchronization. We used live calcium imaging to collective chemosensing monolayers of fibroblasts in response to periodic external ATP stimuli and found that the cells formed a multicellular communication network that gradually evolved and reinforced collective synchronization. We used information-theory to quantify the asymmetric information-transfer between pairs of cells and defined quantitative measures to how single cells receive or transmit information in the multicellular network. We found that cells take different roles in intercellular information-transfer. Initially short-range communication dominates where cells tend to influence and to be influenced by nearby cells. However as the collective become synchronized long-range communication takes the dominant role, when cells are more likely to influence and to be influenced by cells located further apart. We hypothesized that this can be explained by gradual integration of spatial cues that reinforce the roles that cells take in collective synchronization. Indeed, cells maintained their roles in the communication network between consecutive stimuli cycles and reinforced them over time suggesting the existence of a cellular “phenotypic memory”. We identified a subpopulation of cells characterized with higher probability of both receiving and transmitting information. These “communication hub” cells tend to be most stable - not switching to other roles, thus leading to gradual enrichment in communication hubs that is associated with the gradual establishment synchronization. These results suggest that information is spreading more effectively in the multicellular network by gradual enrichment in “communication hubs” to establish effective collective synchronization. An d thus we propose that synchronization emerges from division of labor of the individual cells that gradually reinforces synchronized calcium signaling from the local to the global scale.

P2495/B753

Visualizing Biological Simulations to Improve Access to Simulation for Research and Learning.

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Simulation is a powerful tool for investigating physical and chemical mechanisms that are difficult or impossible to directly observe *in vivo*. However, existing biological simulation software is unintuitive for most researchers to use, does not provide a robust way to visualize and communicate results, and generally does not interoperate. This software often requires access to high performance computing resources and secondary knowledge of technology, including usage of command line interfaces and software development tools and processes. These requirements serve to discourage users who may otherwise be able to use simulation as a tool for research and learning, and result in the exclusion of valuable domain expertise from this field. To fill these gaps, we are building software tools to empower users with domain expertise in biology to build, run, analyze, and share simulations. These tools include a web-based graphical user interface (GUI) for defining and analyzing models, a 3D viewport that visualizes spatial simulation results in real time (when possible) alongside plots of quantitative measurements, and a software layer that integrates existing software packages from prior work in computational biology. To inform the design of these tools, we have conducted expert interviews with

dozens of labs involved in projects combining experimental approaches with computational modeling. A needs analysis of their feedback has informed the design of our first user interfaces and has guided the construction of initial models for nucleating branched actin and growing microtubules. These models will serve as starting points for users to build custom models of cytoskeletal systems with their favorite actin-binding proteins or microtubule-associated proteins. We plan to continue to add more systems to eventually enable multiscale modeling of whole cells. To simulate these models at multiple scales, we are currently using the computational software packages ReaDDy and CytoSim, but plan to add more packages to support other systems. This project, codenamed AgentViz, aims to make biological simulation software easier to access and use in order to facilitate collaboration between experimental and computational biologists, and to provide easy visualization and sharing of results. Tackling this problem requires an interdisciplinary team approach, including expertise in usability, visualization, and software development in addition to domain knowledge in biology.

P2496/B754

The Utilization of Multivariate Data an alysis to Assist in the Production and Scale Up of a Live Virus Based Vaccine.

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The utilization of multivariate data analysis to assist in the production and scale up of a live virus based vaccine Mass production of vaccines hinges upon successful scale-up. Failure to scale-up successfully results in delayed product licensure. Selecting the correct scale-up criteria, such as power input per cubic impeller diameter, was critical to the success of the increasing scale that we will present. Reactor geometry variation prevents consistent scale-up for all criteria. By choosing a power per volume approach, it becomes impossible to scale based upon Kolmogorov eddy length, as eddy length depends upon the chosen power input. Therefore, it is critical to scale up based upon the correct parameter. This work evaluates the production of a live virus by mammalian cells cultured on microcarriers. It explores various volumetric scales, as is demanded to efficiently identify a robust operational space. Metrics of successful scale up include scale comparisons in volumetric potency, specific metabolic uptake rates, specific growth rates, and specific productivity of potent virus particles. All metrics are considered in regard to time and examined through multivariate data analysis. We will report the results of partial least squares multivariate analysis to identify which variables correlate most strongly with potency, specific productivity, and the total particle to infectious particle ratio.

P2497/B755

Development and Application of a Lab Bioimage Informatics System for Fluorescence Microscopy Movies.

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Fluorescence microscopy is a widely used technique in cell and molecular biology. Ongoing advances in microscopes and digital imaging, sample preparation and labeling, fluorescent molecules and proteins, and genome editing are generating datasets of increasing size and complexity. The emerging field of bioimage informatics turns this big bioimaging data into biological insight. We used Fiji/ImageJ, Matlab, and GitHub/Git to build a lab bioimage informatics system that facilitates the organization, processing, and analysis of our fluorescence microscopy movies. To start, each movie is assigned a guaranteed lab-unique, human- and machine-readable identifier. This identifier then functions as a primary key for tab-

delimited text files of metadata, annotations, processing parameters, and analysis results, and also facilitates human- and machine-readable organization of raw movie files and files generated by processing and analyses. Our recently published experimental work demonstrates this system meets three main bioimage informatics goals. First, data are organized in a machine-friendly way. Almost 500 fluorescence microscopy movies, >1TB of raw data, acquired by 4 different people over 4 years, were accessed programmatically and analyzed in a consistent, semi-automated manner. Second, data are machine-readable by multiple programs. Movies were processed and annotated in Fiji/ImageJ, timeseries were analyzed and plotted in Matlab, and statistics were analyzed and plotted in Prism, R, and/or Excel, with frictionless transitions between programs. Third, there is strong data provenance. An y data point can be traced back to its raw movie, with a record of the exact processing and analysis procedures and parameters used. Our bioimage informatics system adds further value by facilitating exploration of the data, which allowed us to see that our experimental biological system responds to the level and activity of a protein of interest with a switch-like response. Our bioimage informatics system has also been used to support multiple collaborations, with cases where we have done the imaging in our lab and provided the output data to our collaborators, as well as cases where we have analyzed imaging data acquired in other labs. Recent research revealed our bioimage informatics effort to be in line with more developed and better supported solutions, including the Open Microscopy Environment (OME), Icy, BisQue, and CellProfiler. Ongoing work is exploring which of these established solutions, or combination of solutions, will best serve the ongoing and future needs of the lab. Once identified, our system will be merged with this preferred solution and optimized for ease of use.

P2498/B756

Morphology Space of Mitochondrial Networks in Budding Yeast: Theory and Experiment.

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Mitochondria form dynamic tubular membrane networks underneath the surface of budding yeast. Little established quantitative language exists to describe the dynamics and structure of mitochondria on a whole-cell scale, which changes over a cell's lifetime due to mitophagy, fission, and fusion. Here, I use tools from graph theory and applied topology to develop a quantitative description of whole-cell mitochondrial morphology. Using this language, I evaluate the applicability of a set of mathematical models to describe the behavior of mitochondria towards developing experimental tests of morphological regulation.

P2499/B757

A Simple Mathematical Model for Branching Morphogenesis.

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The lives of many multicellular organisms depend on an ability to maintain an organized branching structure of fully developed tissues. An imal tissue structure (e.g. lung, breast, kidney, pancreas, neurons, salivary glands, intestine, vasculature) often consists of branched organized systems that arise through the process of “branching morphogenesis.” However, the mechanisms that control branching morphogenesis are not fully elucidated. We hypothesize that to precisely control the organization of branches in tissues, biological rules must exist that explain branching morphogenesis. We modeled different branching patterns based on a specific number of decreasing-sized branches that arise from a

main branch and discovered that specific branching structures are generated based on p-Fibonacci sequences. An agent-based model was then created using NetLogo to show how specific branching organizational patterns can be dynamically maintained in 3D structures that simulate branching patterns found in nature. Understanding of how branching morphogenesis normally occurs in nature might also help explain how branching disorganization occurs in cancer and other diseases.

P2500/B758

Application of Topological Data an alysis Methods in Machine Learning-based TCR Specificity Prediction Models.

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T cells are key players of the adaptive immune system featuring an extremely diverse set of epitope-specific T cell receptors (TCRs). Computational prediction of TCR binding specificity remains one of the central problems of state-of-art adaptive immunity research. Several approaches have been recently proposed to infer TCR specificity and determine TCR specificity groups using primary sequences, structural data, and TCR physicochemical properties (1-3). The performance of the existing methods is significantly limited by the lack of available training datasets. However, machine learning methods may partially compensate for these limitations. Here we used a machine learning model based on topological data analysis approach to define TCR specificities from TCR-epitope specificity data present in the VDJdb database (4). Persistent homology (5) and persistent images (6) techniques were applied to extract features. The classification was performed using the gradient boosting algorithm provided in the XGBoost library. We prepared a synthetic dataset consisting of 3000 CDR3 sequences with a defined specificity to GILGFVFTL (Influenza a virus) peptide and 3000 CDR3 sequences sampled from naïve repertoire as a non-specific control data. The initial evaluation of our model demonstrated performance with ROC AUC score 0.76 and precision 0.75. We conclude that the proposed strategy for feature engineering can substantially boost the performance of TCR specificity prediction methods. 1. Glanville J, Huang H, Nau A, Hatton O, Wagar LE, Rubelt F, et al. Identifying specificity groups in the T cell receptor repertoire. Nature. 2017;547(7661):94-8. 2. Dash P, Fiore-Gartland AJ, Hertz T, Wang GC, Sharma S, Souquette A, et al. Quantifiable predictive features define epitope-specific T cell receptor repertoires. Nature. 2017;547(7661):89-93. 3. Pogorelyy MV, Minervina AA, Shugay M, Chudakov DM, Lebedev YB, Mora T, et al. Detecting T cell receptors involved in immune responses from single repertoire snapshots. PLoS Biol. 2019;17(6):e3000314. 4. Bagaev DV, Vroomans RMA, Samir J, Stervbo U, Rius C, Dolton G, et al. VDJdb in 2019: database extension, new analysis infrastructure and a T-cell receptor motif compendium. Nucleic Acids Res. 2019. 5. Carlsson G. Topology and Data. B Am Math Soc. 2009;46(2):255-308. 6. Adams H, Emerson T, Kirby M, Neville R, Peterson C, Shipman P, et al. Persistence images: a stable vector representation of persistent homology. J Mach Learn Res. 2017;18(1):218-52.

P2501/B759

Computational Fluid Dynamics Model for Cellular Interaction Studies of Sickle Cell Disease Vaso-occlusions in a Microfluidic Device.

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Individuals with sickle cell disease (SCD) suffer from many complications, including organ damage, stroke, and cardiovascular issues. These associated co-morbidities are linked to vaso-occlusions (VOs), chronic blockages within the vasculature. Several factors including sticky, stiffer sickled red blood cells, inflammation, upregulation of adhesion molecules, and increased cell aggregation contribute to VO formation; however the VO mechanism remains elusive. Microfluidics offer the ability to model the vasculature and probe cellular interactions in a non-invasive manner. Vasculopathies such as SCD and the vaso-occlusion phenomenon are ideal for microfluidic modeling. The addition of computational fluid dynamics (CFD) analysis can further provide results which can be used to inform experimental design decisions and potentially extended to clinical studies. Specifically, VO models can provide predictive outcomes for VO occurrences within SCD and identify occlusion prone areas within microfluidic platforms which can be extended to experimental and potentially preclinical studies. We present a CFD blood flow simulation within a branched microfluidic vascular model based on the Carreau model and Murray's law. Several models have been developed to demonstrate variations in blood flow, including, but not limited to, the Carreau, Power Law, and their modifications and derivatives. Based on previous model comparison studies, we found that the Carreau model was a suitable model for our study. We found that VOs form preferentially near bifurcations within 60 s, a timeframe which aligned with occlusion microfluidic studies. Average cellular velocities within the SCD simulation were slower than the healthy study, 1.3 versus 1.5 mm s^{-1}, respectively. Further, the SCD simulation exhibited a deceleration rate of approximately $0.35x^{-1}$ mm s^{-2} (x is time) due to cell adhesion. Velocity profiles and shear rates align with clinical and experimental reports. This study presents a CFD model capable of simulating diseased and healthy blood flow within a branched channel geometry. We assert that results from this model can be utilized to inform experimental and microfluidic system design decisions.

Development and Morphogenesis: Epithelial Development

P2502/B760

Extracellularly Extruded Syntaxin4 Acts as a Novel and Potent Regulator for Dynamic Mammary Epithelial Morphogenesis.

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Mammary epithelium is a unique tissue in that it undergoes distinctive morphogenesis in puberty, pregnancy and lactation periods. Thus, this tissue has been considered as a suitable model to study mechanisms underlying epithelial cell arrangement and complex epithelial morphogenesis. During puberty, the leading edges of the multilayered growing ducts (designated as terminal endbuds, TEBs) simply undergo branching morphogenesis. Upon pregnancy, however, dramatic cell arrangements occur in TEBs to construct cystic structures comprised of an enclosed single cell layer (alveoli). These epithelial morphogenic processes are known to be coordinately controlled by several extracellular signaling molecules, however, their regulation mechanisms remain largely uncovered. Previously, we have shown that a t-SNARE protein syntaxin4 (Stx4) is extruded extracellularly in subpopulation of mammary

epithelial cells and propagates morphologic signals in the nascent cells, leading to an asymmetric tissue organization that resembles branching morphogenesis. Using a polarized mouse mammary epithelial cell line EpH4, we herein show that extracellular Stx4 can also play an important role in alveolar formation. EpH4 cells that have been introduced with a tetracycline inducible Stx4 transgene connected with a signal peptide and T7-tag were rotated on non-adherent surfaces to form TEB-like multicellular cell aggregates, embedded in ECM-rich Matrigel, and cultured with induction of expression of extracellular Stx4. We found that while all the cells expressed Stx4 extracellularly, only inner cell populations in the aggregates, but not those faced to Matrigel, actively migrated outward, integrated into outermost cell layer, and ultimately, the cell aggregates exhibited inflated beautiful cystic structures comprised of an enclosed single cell layer. An alyses with electron microscopy and immunohistochemistry demonstrated that cells in these alveoli-like structures displayed clear apico-basal polarity with tight junctions at the lumen-proximal cell-cell contact sites. We also found that extracellular Stx4 directly interacted with an epithelial adhesion molecule E-cadherin to hinder epithelial cell-cell adhesions. These observations suggest that Stx4 is endowed with the ability to spatially regulate dramatic cellular arrangements for complex epithelial tissue morphogenesis, in coordination with ECM and cell adhesion molecules.

P2503/B761

Morphogenetic Control of Epithelial Topology.

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The epithelium is a fundamental tissue architecture that defines the boundary of many organs. While past studies have proposed how changes in the cell's mechanical properties induce local shape changes, the statistical properties of epithelial tissues have not been addressed. What are the cell biological and physical conditions that determine whether an epithelium remains connected, or divides into multiple, topologically distinct epithelia? to address this issue, we study epithelial morphogenesis by culturing mouse embryonic stem cells as free-floating 3D aggregates and differentiating them to neuroepithelia. Within 4 days, a continuous apical membrane domain forms in the interior of the tissue as a result of collective cell polarization and epithelialization. Treatment with retinoic acid induces the apical membrane to split up into multiple spherical structures, or fluid-filled cysts. We hypothesize that apical surface area and its topology are controlled by retinoic acid-mediated down regulation of PODXL, an apical membrane protein with a negatively charged extracellular domain. Indeed, PODXL heterozygote cells show fragmented apical surfaces in the absence of retinoic acid, and PODXL overexpression show continuous epithelium overcoming the effect of retinoic acid. We develop a biophysical framework that connects the mechanics of epithelial cells to the statistical mechanics of fluid membranes. Our theory allows us to predict epithelial topology from the balance of actomyosin contractility and repulsion of PODXL molecules at the apical membrane. Thus, we elucidate the cell biological basis for retinoic acid-mediated morphogenesis, and propose that epithelial self-organization can be conceptually understood in analogy to how surfactant molecules self-assemble.

P2504/B762

Junctional Epithelium Derived from the Icam1 Positive Papillary Layer Cells.

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Objectives: the continuity of epithelial tissue is collapsed by tooth eruption. The junctional epithelium (JE) is attached to the tooth surface by hemidesmosomes, which constitutes the front-line defense against periodontal bacterial infection. The gingival epithelium consists of the oral gingival epithelium (OGE), oral sulcular epithelium (OSE) and junctional epithelium. Previous our study indicated that JE constitutively expressed ICAM-1. Although JE might be derived from enamel organ, the exact cell source of JE has not been clarified. In this study, we examined the development of JE during the eruption of mouse mandibular molars with the special attention to the expression of ICAM-1. Methods: the mandibles of BALB/c mice from 0-21 day postnatal (21 dPN) were used in this study. Specimens were fixed with 4% paraformaldehyde, decalcified with 10 % EDTA and embedded in Tissue-Tek O.C.T Compound. Frozen sections were processed for the detection of ICAM-1. Results: ICAM-1 positive cells were detected in papillary layer of enamel organs from 12 dPN. At 16 dPN, the tip of the cusp of first mandibular molar located just below the oral epithelium. Ameloblasts differentiated into the reduced ameloblasts which were no more than detected at the tip of the cusp. At the cervical side of the crown, reduced ameloblasts attached to the enamel, which expressed no ICAM-1. The papillary layer cells expressed ICAM-1. At 19 dPN, the first mandibular molar erupted. ICAM-1-negative reduced ameloblasts still attached to the tooth surface. The cells of the papillary layer expressed ICAM-1. At 21 dPN, reduced ameloblasts were disappeared and the papillary layer cells expressing ICAM-1 directly attached to the tooth surface of both mesial and the interdental papillary layer. The epithelial cells of OSE showed no ICAM-1 expression at all. Conclusions: These results indicated that JE developed from the cells in the papillary layer.

P2505/B763

Transcriptional Induction and Mechanochemical Propagation of a Morphogenetic Wave Duringinvagination of the Drosophila Endoderm.

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Morphogenesis of the *Drosophila* posterior endoderm involves complex epithelial reshaping through invagination and a dorsal anterior movement of cells. Rho1 and non-muscle Myosin II-dependent apical constriction drives invagination of the posterior endoderm, but the mechanisms underlying its polarized dynamics remain unknown. We report two phases and modalities of Rho1 and MyoII activation. First, Rho1/MyoII are induced apically in a spatially restricted primordium region via localized transcription of the GPCR ligand Fog. Second, a tissue-scale travelling wave of Rho1/MyoII activation and cell invagination progresses anteriorly across the dorsal epithelium at a constant speed of 1 cell every 3 minutes. The wave does not require sustained gene transcription, and is not governed by regulated Fog delivery. Instead, MyoII inhibition blocked acute Rho1 intracellular activation and its propagation from one cell to the next, revealing a mechanical feedback driven by MyoII. Simulations of a 1D-contractile viscoelastic material suggest that that MyoII contractility provides both local feedback amplification and spatial coupling necessary for wave progression. Finally, we identify a cycle of 3D cell deformations that

link MyoII activation and invagination in one row of cells to vitelline membrane attachment, apical spreading, MyoII activation and invagination in the next row, to drive anterior progression of the invagination wave. Remarkably, the alpha-PS3 integrin Scab mediates apical attachment of cells to the vitelline membrane and is involved in MyoII activation, revealing a role of integrins as potential molecular effectors of the mechanical relay mechanism during wave progression. Thus endoderm morphogenesis emerges from local transcriptional initiation and a mechanically driven travelling cycle of cell contraction and deformation.

P2506/B764

Mechanics of Passive Cell Rearrangement during Epithelial Convergent Extension: Understanding Experimental Observations through Theory.

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Convergent extension (CE) is a critical process in shaping embryos and organs, driving their growth. CE is a common trope in animal morphogenesis for narrowing tissue in one direction and lengthening it in the other. One strategy of CE involves directed cell intercalation (i.e. oriented T1 transitions). For instance, the vertebrate neural epithelium elongates via directed cell intercalation as it forms the neural tube, the precursor of the spinal cord. Failure of this process leads to birth defects such as spina bifida. CE is proposed to be the result of a coordination between biochemical patterning that establishes and maintains the direction of intercalation and mechanical responses from passive and active elements. However, it has been difficult to delineate mechanical from biochemical with traditional experimental design, so we have adopted a computational modeling approach inspired by ongoing studies of *Xenopus* neural CE. The relative contributions from active and passive processes during directed cell intercalation remains unclear. For instance, passive jamming and unjamming transitions have been implicated in epithelial morphogenesis. To understand the role of passive processes, we simulate passive epithelial responses to external CE forces. Such a model isolates the passive responses to tissue field elongation from contributions from active cellular processes. In the model, cells are represented as interacting particles in a square bounding box. Boundaries are moved such that the field of cells undergoes area-conserving CE. Time series of polygonal cell networks are analyzed to quantify deformations, cell neighbor exchanges, and cell area fluctuations. Additionally, we quantify local tissue strain by defining a 2-level *corona* as a cell, its surrounding neighbors, and its neighbors' neighbors. We then analyze the dynamics of the strain experienced by this domain. Recapitulating endogenous rates of *Xenopus* neural CE reveal neighbor exchanges and directional junctional remodeling that mimic in *vivo* observations. Extending our simple simulations by incorporating active cell elements such as actomyosin contractility and junctional remodeling will enable quantitative assessment of specific biochemical and mechanical drivers of tissue shape change.

P2507/B765

Cell-Cell Adhesion Links Cell Shape and Rearrangement Speed during *Drosophila* Axis Elongation.

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During development, simple epithelia reorganize into tissues with complex form and structure. Tissue reorganization during morphogenesis can be rapid. In *Drosophila*, cell rearrangements in the embryonic epithelium double the length of the body axis in 30 minutes. Adhesion at cell-cell contacts, mediated by junctional proteins such as E-cadherin, and contractile tension, generated by actomyosin, are thought to

be key machineries controlling epithelial tissue movements. However, it remains unclear how the balance between adhesion and tension determines epithelial tissue structure and mechanics to control whether tissues remodel and flow like fluids or maintain shape like solids. To gain insight, we systematically modulated the balance between adhesion and tension in *vivo* by using multiple molecular genetics approaches to tune E-cadherin expression in the *Drosophila* embryo. We increased or decreased E-cadherin levels relative to wild type and used live confocal imaging to study the effects on cell shapes and movements before and during axis elongation. Prior to the onset of axis elongation, modulating adhesion levels influenced cell shapes and packings within the germband epithelium. In particular, we found a biphasic response, where either increased or decreased E-cadherin levels at cell-cell junctions was associated with longer cell-cell contacts and cell perimeters. We also found a biphasic dependence of cell rearrangement speed on E-cadherin levels during axis elongation. Interestingly, tissues comprising cells with the longest perimeters also tended to rearrange fastest, consistent with recent vertex model predictions that link cell shape to rearrangement energy barriers and to tissue mechanics and remodeling. To better understand the origins of the observed dependence of cell shape and rearrangement on E-cadherin levels, we also investigated F-actin and myosin II in these embryos, revealing that perturbing E-cadherin levels also influenced the actomyosin contractile machinery. These findings raise the possibility that the coupled effects of E-cadherin on the adhesive and contractile machineries might together impact tissue structure and dynamics and explain the observed behaviors. These systematic, quantitative experimental studies of epithelial tissues structure and mechanics in *vivo* are an essential step in building biophysical models of multicellular tissues and predictive models of morphogenesis.

P2508/B766

Role and Cellular Mechanism of Apico-basal Forces during Tissue Morphogenesis.

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Mechanical forces are critical regulators of cell shape changes and tissue remodelling during developmental morphogenetic processes. within epithelial sheets, planar forces are specifically generated at the apical or basal cell level, while orthogonal forces are developed along the apico-basal cell axis. Although planar forces have been extensively studied, the importance and the cellular machinery required to generate orthogonal forces along the cell apico-basal axis remain elusive. Recently, we revealed that apoptotic cells are generating apico-basal forces that constitute an important mechanical signal involved in epithelial folding in the developing leg of Drosophila. We further identified the cellular mechanism involved and showed that during the initial force-producing stage, cells reorganize their actomyosin cytoskeleton to create a contractile tether connecting the nucleus, which offer resistance to acto-myosin contraction. We then ask if this mechanism was specific to apoptotic cells or could be a general feature of delaminating cells. Interestingly, we revealed that cells undergoing EMT also generate an apico-basal force and that this force constitutes a crucial driving force in mesoderm invagination. Thus, the capability to drive tissue remodelling by generating apico-basal forces could be a general feature of delaminating cells.

P2509/B767

Optogenetic Control of RhoGTPase Activity Reveals Dynamics of Contractile and Adhesive Machinery during Epithelial Morphogenesis.

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Epithelial tissues undergo dramatic changes in shape and structure during development.These changes are driven in large part by contractile forces, which are generated by the cellular actomyosin cytoskeleton and transmitted across the tissue by the cell-cell adhesion machinery. The balance and coupling between contractile forces and adhesion are thought to be key determinants of the mechanical behavior of tissues. A major obstacle to dissecting the mechanisms by which the contractile and adhesive machineries organize and influence morphogenesis has been the lack of tools for flexible and precise manipulation of these machineries in *vivo*. We developed optogenetic tools to modulate actomyosin contractility in the *Drosophila* embryo by controlling the localization of upstream regulators of myosin in the Rho/Rho-kinase signaling pathway, including RhoGEF2. These tools allow for temporally precise perturbations and so are well-suited to studying the dynamics of how patterns of actomyosin activity are established and maintained. We used these tools to perturb Rho activity during germband extension, when forces generated by planar polarized myosin drive oriented cell rearrangements that narrow and elongate the head to tail body axis.We found that optogenetic recruitment of RhoGEF2 uniformly across the apical domain of the tissue is sufficient to disrupt the planar polarized pattern of myosin localization and convert it to a radially polarized pattern. RhoGEF2 recruitment to the apical cell membrane results in a two-step response. First, we observe an initial myosin recruitment to cell-cell junctions, which reduces myosin planar polarity. Second, and rapidly following the first phase, we observe myosin accumulation in the medial-apical domain of cells, which occurs concomitantly with a decrease in junctional myosin and changes in organization of the core adhesion protein E-cadherin. These perturbations to the contractile and adhesive machineries are associated with abnormal changes in apical cell shapes, defects in the orientation of cell rearrangements, and a reduction in tissue elongation. Our results reveal how patterns of RhoGTPase activity influence the spatial and temporal patterns of the contractile and adhesive machineries that drive epithelial morphogenesis and demonstrate the potential of optogenetic approaches for dissecting the dynamics of cellular machinery during morphogenesis in *vivo*.

P2510/B768

Integrin-mediated Attachment of the Blastoderm to the Vitelline Envelope Impacts Gastrulation of Insects.

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Gastrulation is a critical step during the development of multicellular organisms in which a single-layered tissue folds into a multi-layered germband. This shape change is characterized by tissue folding and large-scale tissue flow. The myosin-dependent forces that underlie this process have been increasingly investigated; however, thus far, the possible interaction between the moving tissue and the rigid shell surrounding the embryo has been neglected. Here, we present our quantitative findings on the physical mechanisms governing gastrulation in the red flour beetle *(Tribolium castaneum)*. We investigated the forces expected within the tissue given the myosin distribution observed by multi-view light-sheet microscopy and discovered that an additional external force must be counteracting this

tissue-intrinsic contractility. We then identified that a specific part of the tissue tightly adheres to the outer rigid shell. This attachment is mediated by a specific integrin *(Inflated)* whose knock-down leads to a complete loss of the counter-force. Moreover, in the fruit fly *(Drosophila melanogaster)* knock-down of another integrin *(Scab)* leads to a severe twist of the germband, suggesting that the integrin-mediated interaction between tissue and vitelline envelope may be conserved in insects.

P2511/B769

Planar Cell Polarity and Cell-cell Adhesions Govern Force Production during Embryonic Head-to-tail Axis Extension.

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Convergent extension (CE) is a conserved process of collective cell movement that is essential for head-to-tail axis elongation in animals. Failure of CE is associated with human diseases including neural tube closure defects. Vertebrate CE requires three deeply conserved molecular programs: planar cell polarity (PCP), actomyosin contractility, and cell-cell adhesion. However, it is currently unclear how these molecular programs interface during CE. In this study we used a systems biology approach, including in-vivo super resolution microscopy and tissue-specific mass spectrometry, to determine how PCP, actomyosin contractility, and cell-adhesion are integrated during CE. We found that PCP functioned upstream of the cell-cell adhesion molecule cadherin and PCP was specifically required for polarized localization and cis-dimerization (intracellular dimerization) of cadherin molecules during CE. Next we showed that cis-dimerization of cadherins was required for CE and that disruption of cis-dimerization resulted in axis elongation defects in animals. We then used tissue-specific affinity purification mass spectrometry to identify unique cadherin protein interactions during CE and found a strong interaction with a relatively uncharacterized catenin named Armadillo Repeat Protein Deleted in Velo-Cardio-Facial Syndrome (ARVCF). Finally, we showed that knockdown of ARVCF resulted in mis-localization of contractile actomyosin machinery, tissue scale reduction in force production, and global failure of head-to-tail axis extension. These results connect three deeply conserved molecular programs (PCP, actomyosin contractility, and cell adhesion) during a fundamental developmental process and provide mechanistic insight into the collective cell movements that shape the early embryo.

P2512/B770

Mechanisms of Tubulogenesis in the Sea Star Larva.

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A critical step in organogenesis is the formation of hollow tubes. This process, called tubulogenesis, involves epithelial proliferation, formation of a lumen, size control, distinct cellular polarity, and directional growth. Our aim is to understand the mechanisms of tubulogenesis by use of sea star larvae, a new, tractable model for this prevalent process. Larvae of the sea star Patiria *miniata,* develop two long hollow tubes that bud off the tip of the foregut, grow posteriorly along the length of the digestive system, and contribute to the adult structures at metamorphosis. We followed tube formation in vivo to assess this process through live imaging of actin, microtubules, plasma membranes, and nuclei. We found that tube cells undergo dynamic cell rearrangements and have extensive filopodia, potentially to follow guidance cues coming from the ectoderm. To understand the nature of these cues, we investigated whether specific signaling molecules drive the cellular rearrangements needed for tube

elongation. We found that the VEGF, Notch/Delta, and FGF signaling pathways drive distinct aspects of tube elongation and cell survival, while Wnt signaling drives tube orientation. In summary, our preliminary results establish the sea star larval tubes as a useful in *vivo* model to study cell biological aspects of tubulogenesis, and because of its phylogenetic position as a sister group to chordates, it may shed light on the evolution of vertebrate tubular structures.

P2513/B771

NOVEL Functions of Tight Junction Component Occludin in Vertebrate Epithelium.

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Tight junctions (TJs) are a fundamental feature of both epithelium and endothelium and are indispensable for vertebrate organ formation and homeostasis. However, mice lacking *Occludin* (*Ocln*) develop relatively normal to term. Here, we show that *Ocln* is essential for mammary gland physiology as mutant mice fail to produce milk. Surprisingly, *Ocln* null mammary glands showed intact TJ functions and their epithelial morphogenesis, cell differentiation, and tissue polarity were all normal, suggesting that *Ocln* is not required for these processes. Using single cell transcriptomics, we identified milk-producing cells (MPCs) and found they were progressively more prone to ER stress as protein production exponentially increased during late pregnancy and lactation. Importantly, *Ocln* loss in MPCs resulted in greatly heightened ER stress; this in turn led to increased apoptosis and acute shutdown of protein expression, ultimately leading to lactation failure in the mutant mice. We show that ER stress increase was caused by a secretory failure of milk proteins in *Ocln* null cells. Consistent with a direct role in protein secretion, Occludin resided on secretory vesicles and bound to SNARE proteins. Finally, we show that Occludin is also essential for secretory functions of other epithelial organs. Together, our results demonstrate that *Ocln* play novel functions rather than its traditional roles in TJs biology in vertebrate organs.

P2514/B772

Ecdysone Regulates a Change in Epithelial Barrier Function of *Drosophila* Wing Imaginal Discs during the Third Instar.

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A key function of epithelial tissues is to compartmentalize the body and establish local microenvironments that meet the needs of organs or tissues. The epithelial barrier is regulated in response to changing needs of the organ or tissue. Understanding how the barrier is regulated during development could provide insights into how tissues respond differently to similar stimuli at different points in development. We examined the regulation and change of the epithelial barrier during a period of rapid development: the late third instar of *Drosophila* wing imaginal discs (larval precursors to adult wings). We developed a method to measure barrier permeability relative to genetically or physically disrupted barriers. We determined that by the mid-third instar, wing discs have developed a functional epithelial barrier that becomes more exclusive in the late third instar. To determine the cause of the functional change, we looked at the localization of septate junction components. One core component of septate junctions, Coracle (protein 4.1 homolog), is diffusely localized along the lateral cell membrane during the mid-third instar, but becomes tightly localized to septate junctions as barrier function changes. We determined that Coracle is only necessary for barrier function once localized at the junctions. The timing of the change in barrier function correlates with many developmental transitions,

including the limitation of regenerative ability; these processes are regulated by the steroid hormone ecdysone. To determine if ecdysone regulates changes in barrier function, we artificially increased circulating ecdysone levels by feeding the larvae ecdysone. Larvae fed ecdysone developed a more exclusive barrier earlier in development. We limited ecdysone signaling by locally expressing dominant negative alleles of the ecdysone receptor. This disruption of ecdysone signaling did not affect barrier function in mid-third instar wing discs. However, the barrier failed to transition to the more exclusive, late barrier. Late discs expressing the dominant negatives show diffuse Coracle localization. These data indicate that ecdysone regulates the localization of Coracle, which changes the function of the epithelial barrier. We hypothesized that the changing barrier could sequester signaling peptides in the disc lumen late in development. We assessed this hypothesis with the damage-response peptide *Drosophila* insulin-like peptide 8 (Dilp8). Dilp8 is secreted apically into the lumen but functions in the brain and prothoracic gland to delay development and allow time for regenerative repair. We found that disrupting the epithelial barrier synergistically amplified Dilp8 signaling, indicating that the late barrier may limit Dilp8 by preventing diffusion from the lumen.

P2515/B773

Ecdysone Signaling Shapes Tissue Regeneration through Splice Variants of the Transcription Factor *Broad*.

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As tissues develop, their regenerative capacity is often diminished. In *Drosophila melanogaster* imaginal discs (larval precursors to adult tissues) lose the ability to regenerate near the end of larval development. This loss of regenerative capacity coincides with an increase in systemic levels of the steroid hormone ecdysone, a key coordinator of *Drosophila* developmental progression. Experimentally increasing systemic ecdysone levels by feeding larvae ecdysone limited regeneration that was observed in adult tissues. Surprisingly, high ecdysone levels also increased activation of regeneration genes. This result was scalable: lower concentrations of ecdysone fed showed more activation of regeneration pathways than higher concentrations. Therefore, we hypothesized that low concentrations of ecdysone, such as those found before regeneration restriction, promotes regeneration while higher levels of ecdysone found at the prepupal stage cause changes in disc epithelia that interferes with the activation of regeneration pathways. To determine how ecdysone impacts regenerative signaling, we looked at downstream targets of ecdysone. We find that in wing discs, expression of *broad (br)* splice variants (*Z1-Z4*), which are early targets of prepupal ecdysone signaling, coincides with regeneration restriction. We determined that expression of *br* splice variants are necessary for regeneration restriction. Loss of *br* expression using *br-RNAi* or variant-specific mutants allows for activation of key regeneration genes, *wingless* (*wg*) and *dilp8*, past the regeneration restriction time-point. In addition, loss of *br* allows for increased and extended activation of regeneration pathways. Through the over-expression of the variants early in imaginal disc development, we determined that *brZ1, brZ2 and brZ4,* limit regenerative activity. Interestingly, *brZ3,* activates regeneration pathways. We are currently investigating the mechanism through which *br* variants suppress regeneration and whether they facilitate activation of regeneration pathways at lower ecdysone concentrations. Preliminary data suggests that *br* variants do not regulate JNK signaling in the wing disc but may instead facilitate the epigenetic modifications that lead to suppression of regeneration genes at the end of larval development. Our findings would provide insight into how endocrine signals regulate the regenerative competence of cells.

P2516/B774

Investigating the Principles of Homeostatic Vascular Remodeling Via 4d Imaging of Live Mice.

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Our organs depend on an organized vascular network that supplies them with nutrients and soluble factors via the bloodstream. The endothelial cells (ECs) that line our blood vessels dictate crucial aspects of vascular function and morphogenesis. However, there are still many open questions concerning the cellular mechanisms by which the vasculature is able to maintain homeostatic conditions. To this end, we are investigating how the vascular network 1) is able to adapt to organismal growth to reach its steady state and 2) once established, how does it keep functioning despite many insults including injury. The challenge in addressing these questions is the inability to follow the same tissues and cells over time. To overcome this roadblock, we have adapted our intravital imaging of skin epithelium to now track and manipulate endothelial cells in live mice. We have observed that the branching architecture of the dermal vascular plexus is established early during neonatal growth, with capillary loops uniformly expanding in coordination with tissue growth. Tracking of individually labeled cells reveals that the vast majority of capillary ECs undergo cell elongation, suggesting a role for this behavior for coordinated loop expansion. Interestingly, ECs were also observed to participate in migration within lumenized blood vessels during this period, a phenomenon that is reminiscent of embryonic stages. Longitudinal tracking of cells over several weeks shows that the rate of EC migration is largely diminished as the animal reaches adulthood. Genetic manipulation of Rac1 in ECs during neonatal growth to perturb luminal migration leads to defects in vessel branching and coverage. As the plexus size and cell behaviors reach stability, we next sought to understand the mechanisms by which ECs are able to cope with injury and maintain vascular integrity. By carrying out targeted laser ablations to inflict local damage at the individual cell level, we find that ECs do not respond with proliferation but instead elongate towards the site of damage. Intriguingly, we also observe that injury in the periphery of individual ECs results in a pinching off and discarding of damaged membrane in what is likely an exocytic mechanism of self repair. This is a phenomenon that has not previously been described in ECs and we are currently investigating the role that caveolae (enriched in ECs) could be playing in the self repair process by utilizing Caveolin-1 knockout mice. Overall, this study sheds light upon fundamental mechanisms by which ECs maintain and control vascular homeostasis.

P2517/B775

Zbp1 Expression Is Required to Support Epithelial Cyst Formation and Tissue Morphogenesis.

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It is well-appreciated that Zipcode Binding Protein-1 and its regulatory action on mRNA stability, localization, and translation are essential for normal development. For example, loss of ZBP1 expression and activity has been linked to dysfunctional chemotactic activity and metastasis. Previously, Gutierrez et al. (2014) documented the importance of localized β-actin expression, under the control of ZBP1, on the formation of adherens junction in cultures of MDCK epithelial cells. Delocalizing β-actin translation resulted in delayed assembly of the adherens junction complex and increased barrier permeability. ZBP1 has also been shown to regulate the localization and translation of E-cadherin as well as the stability of β-catenin mRNAs. Thus, ZBP1 appears to impact the formation and function of adherens junctions at

multiple physiological levels. The current study seeks to investigate the consequences of ZBP1 expression on adherens junction formation, barrier function, and epithelial cyst morphogenesis. In order to address this question, stable MDCK ZBP1 knockdown cell lines were generated using shRNA. Following ZBP1 knockdown, E-cadherin and β-catenin levels are reduced, and confocal immunofluorescence imaging identified the presence of "holes" in monolayers due to incomplete adherens junction formation. Using cell aggregation and fluorescent dextran permeability assays, MDCK ZBP1 knockdown cells exhibited slower rates of aggregation, decreased aggregate size, and increased barrier permeability. When cultured in Matrigel, MDCK cells form cysts with highly defined apical and lateral surfaces with a single lumen. Upon ZBP1 knockdown, MDCK cysts demonstrated disrupted adherens junction organization, increased number of lumens, increased barrier permeability, and altered orientation of mitotic spindles. MDCK cells expressing β-actin mRNA lacking the 3'UTR zipcode sequence recapitulated the aforementioned phenotypes observed by ZBP1 knockdown. Thus, ZBP1 appears to coordinate localization and translation of multiple adherens junction components that have direct effect on epithelial barrier function, spindle orientation, and apical-basal polarity.

P2518/B776

Axolotl MARCKS Like Protein Is Extracellularly Released Via a Non-canonical Protein Secretion Pathway.

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How do regenerative animals start regeneration? a key feature of complex appendage (limb and tail) regeneration in the axolotl (*Ambystoma mexicanum*) is the accrual of a proliferative zone of progenitor cells at the injury site, a structure called the blastema. Blastema formation and proliferation is dependent on epidermal cells, which migrate in to cover the amputation plane. This newly formed epidermal layer is called the wound epidermis (WE). It was already demonstrated by Tornier in 1906 that substitution of the WE by a graft of uninjured epidermis arrested regeneration showing that the WE is an essential tissue to promote the process of appendage regeneration in salamanders (Tornier, G. 1906. *Arch. fur Entwmech*). However, it had been remained unclear what injury-related signals from the WE trigger blastema formation. To identify extracellular factors that initiate proliferation in blastema cells during regeneration, we took a functional expression cloning approach. We identified Axolotl MARCKS Like Protein (AxMLP) as an extracellular molecule that induces cell cycle in cultured myotubes. Gain- and loss-of-function assays in *vitro and* in *vivo* strongly suggested that AxMLP triggers the initial proliferative response after injury (Sugiura, T., et al., *Nature* 2016). Importantly, we found that AxMLP was expressed in the WE and AxMLP was released into extracellular space in *vitro*. This was surprising, as MARCKS family proteins had been described to be intracellular proteins (El Amri, M., et al., *J Biomed Sci.* 2018). Indeed, MARCKS family proteins (including AxMLP) do not have a canonical secretion signal sequence. In the current study, we focus on the mechanisms of AxMLP secretion. Our preliminary results suggest that: 1, AxMLP is not secreted via the canonical secretion pathway 2, Extracellular vesicles containing AxMLP can induce proliferation of cultured myotubes 3, Membrane association of AxMLP is related to its secretion Taken together with our previous study, these results imply that MLP released extracellularly from the WE via a non-canonical protein secretion pathway stimulates progenitor cell proliferation and eventually contributes to forming the blastema. We are now performing a drug screen to identify the molecules that regulate AxMLP secretion.

P2519/B777

Deciphering How Spatial Patterning of Radial Cell Fates Drives Hair Follicle Morphogenesis.

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During embryonic development, signaling pathways are activated in spatially-defined patterns that specify new cell fates and drive tissue morphogenesis. The mammalian epidermis, which is decorated with thousands of hair follicles arranged in a periodic pattern, is an ideal model system to investigate how spatial patterning drives cell shape and motility changes that underlie epithelial morphogenesis. Periodically-patterned Wnt and Shh signaling specify early hair placodes, which are comprised of two, radially-arranged cell types that undergo extensive cell rearrangements to form polarized epithelial buds. Although both cell types are required for placode morphogenesis, their individual contributions to this stereotyped cell motion are unknown. Here we show that Wnt signaling directs changes in cell-cell adhesion in developing hair placodes and investigate how reciprocal gradients of classical cadherin expression contribute to placode morphogenesis. Ongoing work is testing the necessity of these cell adhesion differences for rearrangements using long-term live imaging of placodes from embryos lacking either E-Cadherin or P-Cadherin.

P2520/B778

Targeting Filopodia during Pattern Formation.

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The ability of Notch signaling to drive the formation of a broad range of biological patterns relies, in many systems, on the activity of cellular protrusions that allow contact between cells at a distance. One example of this is the patterning of sensory bristles on the thorax of the fruit fly Drosophila melanogaster. In this tissue, cellular protrusions are thin, dynamic, actin-based, filopodia-like structures which extend from the basal surface of the patterning epithelia. A challenge in the study of the role of these protrusions is the lack of a specific genetic tool that allows for perturbation of protrusions without simultaneous perturbation of other critical actin-based cell structures and processes. An unconventional myosin, Myosin XV, has previously been shown to localize to, and play a role in the dynamics of, filopodia in Drosophila as well as in other cellular protrusions in mammals, including stereocilia. We hypothesized that specific manipulation of cell protrusions via Myosin XV would lead to changes in the pattern of Notch signaling and bristle patterning in vivo. Using a combination of genetics, cell biology, and confocal imaging, we observed that expression of Myosin XV RNAi leads to increased density of sensory bristles. Consistent with this finding, we observe changes in filopodia dynamics. Finally, we observe that Notch signaling is affected by specific targeting of filopodia, through the use of a transcriptional reporter of Notch signaling. Together these results support a role for filopodia in lateral inhibition during bristle patterning.

P2521/B779

Dia1 Coordinates Differentiation and Cell Sorting in a Stratified Epithelium.

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Prior studies implicate the actin nucleator, Dia1, in shaping cellular adhesions. However, few approach Dia1 and adhesion in terms of cell sorting. Dia1 localizes to the basal layer of stratified mammalian

epidermis, a tissue which relies on cell sorting to maintain its multilayered architecture. Specifically, induction of differentiation in basal keratinocytes coincides with apical movement into suprabasal compartments. In 3D skin cultures, we found that genetic depletion of Dia1 resulted in a poorly packed basal layer. Dia1-deficiency did not prevent stratification but did perturb expression of suprabasal differentiation markers. Since crowding is known to induce differentiation and initiate basal layer egress, we hypothesized that Dia1 prevents keratinocytes from escaping the basal layer before reaching densities amenable to inducing differentiation. Consistent with this hypothesis, we found that forced crowding of Dia1-deficient cells rescued transcriptional abnormalities. Dia1 both supported cortical tension and, paradoxically, accelerated vertical expansion of nascent, intercellular contacts. In aggregation assays, this feature drove Dia1 positive and negative cells into distinct stratified layers in a ROCK-dependent manner. Our results suggested that as basal cells proliferate, Dia1 expression favors reintegration with other Dia1 positive basal cells, in effect, opposing dissemination into the Dia1 negative, suprabasal compartment. Though linked to ulceration and carcinoma, this study illuminates the scarcely explored role of formins in normal physiological processes leading to tissue stratification.

P2522/B780

Utilizing Single-cell Sequencing Technology to Improve Synthetic Skin Equivalents.

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Organotypic culture systems are artificial environments designed to model complex 3D biological systems more accurately than 2D cell cultures. They provide a means to study cellular behavior with more physiological relevance than cell lines while offering higher throughput than in vivo models. While these organotypic cultures are powerful tools, it is important to know which aspects of them are biologically relevant. In our lab, we have performed single cell RNA-sequencing on fully stratified human epidermal tissue that was generated using multiple distinct organotypic culture system protocols. Sequencing experiments coupled with molecular and histological staining elucidated the similarities and differences between in vivo human epidermis and our skin equivalents grown in vitro and provided insight into the cell-cell signaling between different cell types. Both homologous and heterologous clusters of cells exist between normal human epidermis and our skin culture systems. Alterations to the organotypic skin culture were made to abate these distinctions and further sequencing experiments were performed to evaluate these changes. This study moves us closer to generating skin organoids that accurately mimics in vivo human skin which can be used in genetic experiments, drug screens, and ultimately transplantation.

P2523/B781

Developmental Transitions in Epithelial Cell Behaviour Drive Skin Morphogenesis.

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A key feature of animal embryogenesis is the formation of a protective, fluid-retaining permeability barrier. In mammals, the skin epidermis, a tissue comprised of stratified layers of specialized epithelial cells, fulfils this function. In the mouse embryo, epidermal development begins at E9.5 from a single layer of progenitor cells. To initiate the transition to a stratified epithelium, cell divisions within the monolayer are increasingly skewed towards axes of division that are oriented perpendicular to the underlying basement membrane, such that one daughter cell populates an emerging “suprabasal” layer

on top of the preexisting “basal” layer. Consistent with others in the field, we find that until E14.5, the probability of a basal cell to divide perpendicularly positively correlates with increased cell crowding; regions of greater epithelial crowding stratify more quickly than less crowded regions. This phase of cell density-driven stratification precedes differentiation and does not depend on oriented localization of classical asymmetric cell division (ACD) machinery components such as LGN. However, by E15.5, a correlation between cell density and axis of division is no longer observed and stratification is governed by ACD-machinery dependent control. Intriguingly, we previously discovered that E15.5 also represents a developmental transition in modes of loser cell elimination during cell competition in the skin. Before E15.5, loser cells are removed by apoptosis and subsequent engulfment by their epidermal neighbours. However, from E15.5 onwards, losers no longer undergo apoptosis, but instead exit the basal layer via ACD and differentiation. We hypothesize that these two transitions, in stratification mechanisms and in loser cell elimination mechanisms, are linked. Using functional genetics, immunofluorescence and time-lapse imaging, we systematically test a series of candidate genes that may govern this transition. Our candidates fall into two categories: (1) genes that influence the basal cell microenvironment such as adhesion to the underlying extracellular matrix, and the ECM itself; (2) genes associated with the early stages of differentiation in the overlying suprabasal layer that directly contacts the basal layer. Our study will provide fundamental insights into our understanding of how stratified barrier tissues are built in the embryo. Moreover, in epithelial barrier tissues such as the skin that continue to turnover throughout the life of the organism, we speculate that such early developmental transitions are ultimately important for and part of the eventual transition to homeostasis that must occur to both maintain tissue integrity and prevent overgrowth once development is complete.

P2524/B782

Investigating Mechanisms Regulating Cell Adhesion during Tissue Remodeling.

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Proper regulation of cell-cell adhesion is crucial for embryonic development and tissue homeostasis: dysregulation of adhesion during development leads to failures in morphogenesis that produce congenital disorders, and abnormal activation of signals that regulate adhesion in tumors can result in epithelial-mesenchymal transition (EMT) and cancer metastasis. In epithelial tissues, cells adhere to each other through Adherens Junctions (AJs) containing the cell adhesion molecule E-Cadherin. The transcription factor Snail is a conserved regulator of cell adhesion and cell motility that was first identified as a necessary factor for promoting cell motility and EMT during gastrulation in the fruit fly, *Drosophila melanogaster*. Snail has since been shown to repress E-cadherin transcription in many model organisms, and the ectopic expression of Snail in cell culture is thought to promote precocious by reducing adhesion through transcriptional silencing of E-cadherin. Interestingly, recent observations have shown that Snail regulates the stability and localization of AJs independent of transcriptional regulation of E-Cadherin levels. However, the specific mechanism by which Snail controls AJ organization remains unknown, highlighting an important gap in our understanding of the signals that regulate adhesion and govern the cellular decision to undergo EMT. We have begun to investigate this process in the *Drosophila* embryo using a combination of biochemical, cell biological, and genetic approaches to define this mechanism. Here, we present preliminary evidence from the naturally occurring EMT of the *Drosophila* mesoderm during gastrulation, as well as from an ectopically induced EMT in an embryonic epithelium.

P2525/B783

Role of the Arrangement of Basal Bodies in Establishment of the Coordinated Ciliary Beating in Multi-ciliated Cells.

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Multiciliated cells (MCCs) promote fluid flow through coordinated ciliary beating. Tracheal MCCs contain large numbers of basal bodies (BBs), which are short cylindrical structures at the bases of cilia that are docked to the apical membrane. The orientation of BBs, identified by their asymmetrically associated basal feet (BF), is precisely coordinated to support efficient mucociliary transport. In our previous work, we developed a long-term and high-resolution live cell imaging system by using super-resolution microscopy to observe green fluorescent protein (GFP)-centrin2 labeled BBs in mouse tracheal epithelial cells (MTECs). This system revealed stereotypical patterns of BBs and we constructed a theoretical model which indicated that the apical cytoskeleton provides a self-organizing mechanism to align BBs linearly in tracheal MCCs. However, the biological relevance of the alignment and orientation of BBs is still unknown. Here, we examined the mechanism to coordinate the alignment and orientation of BBs and found some pattern of coordination of BBs' orientation in relation to BBs' alignment through apical cytoskeleton network in a PCP-dependent manner. These understanding in coordinated ciliary beating provides a deep insight into how mucociliaty transport is established and how its defects might cause disease.

P2526/B784

Dorsal Closure in Numbers: Quantification of Epithelial Cell Oscillations Using Deep Learning.

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Dorsal closure in *Drosophila melanogaster* embryos is a key model system for cell sheet morphogenesis and wound healing. Multiple sub-systems are involved in the mechanical closing of the dorsal opening. Understanding system dynamics, regulation and causal relations requires a quantitative understanding of the mesoscopic mechanical and dynamic properties of this "active soft material". Individual cells in the amnioserosa, a one-cell-thick sheet of epithelial cells filling the dorsal opening, show sustained oscillations of apical cell area. These oscillations exhibit large variations from cell to cell and during the course of closure. Past studies of epithelial dynamics were restricted to semi-manual segmentation of cell shapes and thus suffered from relatively low statistics. We present a novel analysis pipeline, based on a convolutional neural network (deep learning), that allows an automated and robust segmentation of large numbers of video recordings. We further employ statistical approaches to analyze spatial-temporal dynamics and quantify embryo-to-embryo variability. We observe emerging long-range dynamical patterns providing clues about possible communication mechanisms between cells.

P2527/B785

Microtubules Coordinate Collective Cell Migration in the Developing Mammary Epithelium.

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Branching morphogenesis closely coordinates cell proliferation and collective cell migration to expand tubular epithelial structures. In the mammary gland, early branching relies on cell proliferation to establish a stratified Terminal End Bud (TEB). Once this unit forms, coordinated migration of TEB cells acutely drives elongation and branching. To investigate the dynamic contributions of microtubules to mammary epithelium, we use three-dimensional cultures of mammary organoids--epithelial cell clusters embedded within extracellular matrix. This culture system recapitulates the events of mammary development in a controlled and observable environment. We demonstrate that microtubule targeting drugs paclitaxel, nocodazole, and colchicine acutely arrest elongating branches and that this effect is independent of their effects on mitosis. Tracking individual cells in active TEBs through time-lapse confocal microscopy reveals that stabilizing microtubules with paclitaxel reduces cell speed while destabilizing microtubules with nocodazole reduces cell directionality. A dynamic microtubule cytoskeleton is therefore required for collectively migrating epithelial cells to maintain their speed and direction in 3D substrates. Time-lapse confocal microscopy of microtubule and organelle dynamics in TEB cells indicate that microtubules support the reorganization of epithelial cells for collective migration. These results offer novel insights into the role that the interphase microtubule cytoskeleton plays in collective migration and mammary epithelial morphogenesis.

Stem Cells-Pluripotent Stem Cells

P2528/B786

Secretion of Cardioprotective Factors from Cardiac Colonies Derived from Germline Pluripotent Stem Cells to Combat Myocardial Fibrosis [Samiksha Mahapatra, Breanna Brownson, Conor Dillon,Dr. G. Ian Gallicano]

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Stem cell research for treating or curing ischemic heart disease has, to date, culminated in identifying which scenario is more important; 1) stem cell differentiation into cardiomyocytes that integrate electrically with the heart, 2) stem cells that secrete paracrine factors that promote healing, or 3) combination of both. Following myocardial injury, cardiomyocytes are replaced by extracellular matrix proteins secreted by activated human cardiac fibroblasts that rapidly proliferate eventually forming a mature scar in the infarct area. This process is initially reparative but its long-term effect contributes to fibrosis and myocardial dysfunction. In our work, we consistently found that unipotent germline stem cells, when removed from their niche and cultured in the correct medium endogenously express pluripotency genes, which induce them to become human germline pluripotent stem cells (hgPSCs). These cells are then capable of producing cell types from all three germ layers. Using hgPSCs along with a modified version of a relatively novel cell-expansion culture methodology to induce quick, indefinite expansion of normally slow growing hgPSCs, it was possible to test the potential of cardiac colonies derived from hgPSCs for treating myocardial fibrosis following injury. Upon differentiation into cardiac lineages, our data consistently showed that they not only express cardiac genes, but also express cardio-protective paracrine factors. Our data also suggests that cardio-protective secreting cardiac colonies fuse and integrate with the surrounding tissue via gap junctions in *vivo.* Taking these data a step further, we found that these cardioprotective factors affect human cardiac fibroblasts (HCFs) and human

cardiomyocytes (HCMs) by suppressing *activation* of HCFs. More importantly, we found that these factors induce significant migration/proliferation of HCMs into the infarct zone. Our data suggest that secreted cardio-protective paracrine factors from hgPS-derived cardiac colonies could help tip the balance from fibrosis towards myocardial repair. Note, while the work presented here was based on testes-derived hgPSCs, data from other laboratories have shown that ovaries contain very similar types of stem cells that can give rise to hgPSCs. As a result, hgPSCs should be considered a viable option for eventual use in patients, male or female, with ischemic heart disease.

P2529/B787

Modelling Naa15 Haploinsufficiency, a Cause of Congenital Heart Disease, Induced Pluripotent Stem Cells.

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To better understand the contribution of the NatA complex, responsible for amino terminal (Nt) protein acetylation, to congenital heart disease we characterized NAA15 sequence variants in 4511 congenital heart disease (CHD) probands and genetically engineered isogenic induced pluripotent stem (iPS) cells. 4 loss-of-function, LOF, and one damaging missense (R276W) de novo NAA15 variants and 15 rare inherited missense mutations were found in this cohort. Haploinsufficent (heterozygous LOF), null (compound heterozygous LOF) and NAA15 missense carrying iPS cells were constructed using CRISPR/Cas9 technology to create cell-based models of NAA15-deficiency where the effects of these mutations on Nt acetylation could be evaluated. Haploinsufficent NAA15 iPS cells could differentiate into cardiomyocytes, but NAA15-null iPS cells could not, presumably due to alterations in both the amount and composition in NatA complex (documented by western blot analyses). Although nearly 80% of proteins detected by mass spectrometry have partial or complete Nt-acetylation, Nt acetylation of only 32 and 9 proteins differed in null and haploinsufficient NAA15 cells, respectively. Levels of more than 560 proteins were altered in mutant cells compared to wildtype cells. While most Nt acetylated proteins are fully acetylated, ~30 proteins are partially acetylated; NAA15-haploinsufficiency abrogated Nt-acetylation of 5 of these proteins but did not affect the acetylation of the other 25 proteins. Similar changes were observed in NAA15 R276W iPS cells. These studies define human proteins requiring a complete NAA15 complement for normal activity; deficiencies in one or more of these NAA15 target proteins are likely required for normal cardiac development.

P2530/B788

Mitophagy-induced Release of the Mitochondrial Phosphatase PGAM5 Activates a Nuclear Wnt Signaling Feedback Loop in Differentiating Human Induced Pluripotent Stem Cells.

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Background: Stem cells undergo metabolic reprogramming as they transition from a predominately glycolytic state during pluripotency towards enhanced mitochondrial respiration as they differentiate. The GTPase Mitofusin 2 (Mfn2) is a key mediator of mitophagy, the autophagic degradation of dysfunctional mitochondria. We hypothesized that mitophagy coupled with compensatory biogenesis of new mitochondria could serve as a key mediator of metabolic reprogramming during stem cell differentiation and that mitophagy may also further stabilize the differentiation process. The mitochondrial phosphatase PGAM5 is cleaved upon mitophagy and modulates Wnt signaling in the nucleus, thus providing a mitochondrial-nucleus crosstalk mechanism. We assessed whether cleaved

PGAM5 modulated Wnt/β-catenin signaling to direct stem cell differentiation. ***Methods and Results:*** Human induced pluripotent stem cells were differentiated towards a mesodermal-endothelial cell lineage. In the early stages of mesodermal differentiation, mitophagy doubled as assessed by the colocalization of mitochondria and activated lysosomes from 6.2% to 14.5% (p=0.004). Following this initial burst of mitophagy, mitochondrial mass subsequently increased in differentiated cells as measured by immunofluorescence for the mitochondrial membrane protein TOM20 from (from a mean of 162 to 307 mitochondrial pixels per visual field, p=0.003). Genetic inhibition of mitophagy by shRNA depletion of Mfn2 reduced the efficiency of differentiation by 50% as assessed by qPCR and flow cytometry of the mature endothelial markers VE-cadherin and CD31 (PECAM), indicating the essential role of mitophagy in differentiation. The mitochondrial phosphatase PGAM5, which is cleaved during mitophagy and modulates the Wnt pathway, increased three-fold after the induction of mitophagy on day 4 of differentiation ($p<0.05$). However, shRNA depletion of Mfn2 prevented the increase in PGAM5. Concomitantly, there was an increase in Wnt signaling on day 4 as seen in a doubling of the nuclear localization of β-catenin. Immunoprecipitation demonstrated a direct interaction between β-catenin and cleaved PGAM5 on day 4 of differentiation. ***Conclusion:*** Mitophagy is required for the metabolic reprogramming of differentiating human pluripotent stem cells. Importantly, mitophagy releases the Wnt signaling modulator PGAM5 which regulates the differentiation process and the biogenesis of new mitochondria. This novel feedback mechanism of mitochondrial-nuclear crosstalk in differentiating pluripotent stem cells provides new insights into the intersection of metabolic and developmental signaling.

P2531/B789

Establishment of Culture Method for Intestinal Stem Cells Derived from Human Ips Cells and Applications of Enterocytes Derived from Human Ips Cells for Drug Development.

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The small intestine plays an important role in the pharmacokinetics of orally administered drugs due to the presence of drug transporters and metabolizing enzymes. Enterocytes differentiated from human iPS cells are useful cells for investigating intestinal pharmacokinetics. However, there is a problem with the stable supply. Hence, as a solution to this issue, we attempted to establish a new two-dimensional culture method to maintain intestinal stem cells (ISCs) derived from human iPS cells. Furthermore, we evaluated whether they have a potential to differentiate into enterocytes exhibiting appropriate pharmacokinetic functions. In established culture method, we succeeded in maintaining stemness of ISCs by activating the signaling pathway necessary for maintaining stemness, such as Wnt and Notch signaling. Furthermore, our results verified that the differentiated enterocytes from the maintained ISCs demonstrated appropriate pharmacokinetics functions. We also confirmed the presence of microvilli, which is characteristic structures of the small intestine. In addition, we investigated the use of enterocytes derived from human iPS cells for toxicity evaluation. The main component of intestinal mucus is mucin 2 (MUC2) secreted from goblet cells. MUC2 expression level in enterocytes derived from human iPS cells is comparable to that in the human small intestine. We evaluated drug-induced mucosal damage and protective effects using changes in MUC2 expression as an indicator. We examined changes in MUC2 mRNA expression in enterocytes by non-steroidal anti-inflammatory drugs (NSAIDs) and mucosal protective drugs (MPAs) as model drugs. As a result, NSAIDs decreased MUC2 expression level in enterocytes, whereas MPAs increased MUC2 expression level. In the present study, expression levels

of cytokines such as IL-1β, NF-κB, and TNF-α were changed by NSAIDs and MPAs, therefore, the cytokines were considered to be involved in MUC2 expression changes in enterocytes. In conclusion, the intestinal stem cells maintained by developed culture method was shown to be capable of differentiation into the intestinal cells having pharmacokinetic functions. In addition, by using MUC2 as an indicator, it is possible to evaluate the mucosal damage and protective effect of drugs, which may contribute to pharmacological efficacy and safety of novel drug development research.

P2532/B790

Isolation of Clonal Genome Edited Dual-fluorescent Reporter Human Stem Cell Populations Without Single Cell Cloning.

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The ability to translate human stem cell research results into therapeutic products is burdened by several factors including uncertainty about the influence of stem cell culture reagents, protocols and procedures. Results from stem cell laboratory investigations tend to be difficult to evaluate and compare to results from other labs, and clear correlations between culture conditions and cell state and fate are difficult to discern. In an effort to better track the correlations between culture conditions and stem cell response, we have initiated a program to improve our basic understanding of induced pluripotent stem cell (iPSC) growth and to build predictive models by defining the critical measurable parameters that regulate the maintenance of iPSC pluripotency and differentiation. This effort is dependent on the creation of well-characterized dual-fluorescent reporter stem cell lines that accurately report the transcriptional expression patterns of three transcription factors (TF) known to be regulate pluripotency and transitional cell states during differentiation in human stem cells, OCT4, SOX2, and NANOG. A challenge to this effort is the poor survival of stem cells passaged at low seeding density, which makes the generation of clonal cultures from single cells highly problematic. Our work flow was designed to address this problem. WTC-11 cells were transfected in the presence of TF-specific gRNA-cas9 ribonuclear protein (RNP) complex and “donor” plasmids with homology arms to drive gene specific recombination. The “donor” plasmids are designed to replace the stop codon with a translational read-through signal (P2A) and a nuclear localization signal-sequence (NLS) upstream of a fluorescent protein (GFP or m-Cherry) encoding sequence. Thus, the expression of the fluorescent reporter is regulated by the endogenous control elements and the normal expression of the TF is unaffected. After testing fourteen transfection conditions, we chose a protocol that provide us with ~.01% efficiency of insertion of fluorescent protein. This relatively low efficiency is convenient for our next steps. We distribute post-transfection cells into 96 well plates together with untransfected helper cells to bring the final concentration of cells to ~12,000 cells/ cm^2. Starting three days post-transfection, entire wells are imaged with bright field and fluorescence optics using a very low level of excitation light. Genome editing events are clearly identifiable as clusters of small numbers of fluorescent cells and wells containing single events are noted for further expansion. Using this approach, we have generated clonal dual-fluorescent reporter stem cells without the necessity of single cell cloning.

P2533/B791

Role of Small Molecules in Generation of Cord Blood-Derived Pluripotent Stem Cells.

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In 2006 Takahashi and Yamanaka developed a ground-breaking technology in the field of nuclear reprogramming which doesn't rely on fusion or enucleation of ES cells. They found that differentiated somatic cells can be reprogrammed to a pluripotent state by a set of defined transcription factors (also called Yamanaka Factors). Forced expression of Oct-4, KLF, Sox-2 and c-myc (OKSM) would reprogram somatic cells into pluripotent like stem cells. The resulting cells are named as induced pluripotent stem cells (iPSCs). The term pluripotency means the cells capable of differentiate into all types of cells lineages. Technical and ethical issues hindered the medical use of somatic cell nuclear transfer and embryonic stem cells. Therefore, induced pluripotent stem cells emerged as a powerful technique with great potential for clinical applications. iPSCs were generated by forced expression of Transcription Factor (TFs)Oct4, KLF4, SOX2, and Myc-c from skin fibroblasts. Recently, iPSC has been generated more rapidly and efficiently and the only hurdle which comes across is the rate of conversion of these cells is very slow. In this study, we have initroduced an easy way to reprogram cord blood stem cells using combination of small-molecules in a feeder-free system. Small Molecules carries a unique property which directly or indirectly helps in maintaining pluripotency of stem cells. The development of the integration-free method in a feeder-free system offers a great potential in clinics, drug testing and other applications.

P2534/B792

Membrane Tension Regulates Fgf Driven Fate Choice in Embryonic Stem Cells.

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Changes in cell shape and mechanics frequently accompany cell fate transitions. Yet how mechanics affects the regulatory pathways controlling cell fate is poorly understood. To probe the interplay between shape, mechanics and fate, we used embryonic stem (ES) cells, which spread as they undergo early differentiation. We found that this spreading is regulated by a decrease in plasma membrane tension, and preventing this decrease obstructs early differentiation of ES cells. Moreover, we found that if cell membrane tension is not decreased, endocytosis of FGF signaling components, which direct the exit from the ES cell state, is significantly inhibited. Strikingly, the early differentiation defects we observed can be rescued by increasing Rab5a-facilitated endocytosis. Thus, we show that a mechanically-triggered increase in endocytosis regulates fate transitions. Our findings are of fundamental importance for understanding how cell mechanics regulates biochemical signaling, and therefore cell fate.

P2535/B793

Recreating a Novel 3D Cellular Microenvironment of Human Joint Tissue to Understand the Mechanism of Cellular Senescence in Aged Stem Cells.
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Physiological aging in human is linked to cellular aging which is associated with oxidative stress, genetic instability, telomere shortening, mitochondrial dysfunction, and DNA mutation in some genes that result in premature aging. Age-related changes due to cellular aging in joint lead to degenerative bone disease such as osteoarthritis (OA). Although past studies noted that accumulation of senescent cells in cartilage causes early onset of OA in response to injury or aging, less is known about the interaction of senescent cells with neighboring cells within the local and distant tissues that forms articular cartilage and synovial joint. Here, the study investigates how high level of cellular senescence negatively influence the differentiation commitment of local stem cells for terminal differentiation into chondrogenic/ osteogenic lineages, and develop ineffective communication between different cell types within 3D reconstructed joint tissue. To understand the cellular interactions between senescent cells and neighboring non-senescent cells in human joint tissue, we designed the system to co-culture gradient of 4 different cell types that are encapsulated in hydrogel scaffold, and cultured in the bioreactor. This study uses cell lines derived from induced pluripotent stem cells (iPSCs) to generate gradient of cell layers with consistent genetic background. Cylindrical form of collagen scaffold is cross-linked to compose 3 major layers embedding senescent and non-senescent iPSC-osteoblasts/ osteocytes, iPSC-chondrocytes, and synoviocytes. This cell-embedded scaffold is cultured in tri-chamber bioreactor with osteogenic, chondrogenic, and synovial cell culture media, respectively. The design allows the nutrient flow for corresponding layer section, and gradual fusion of different cell layers through collagen construct. This novel method of co-culturing three cell lines allows to recapitulate cellular microenvironment of human joint, and to further study the role of senescent cells in altering joint tissue microenvironment, which later progresses to OA.

Cell Fate Determination 2

P2536/B794
Overexpression of ROR2 in WNT1 Producing Cells Augments Signaling.
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Morphogens, such as Wnts, are signaling molecules that play a vital role in the early stages of development. Their graded distribution pattern promotes the specification and differentiation of multiple cell types. While the effects on cell specification and differentiation are evident, the Wnt gradient has yet to be directly visualized. Adding to the ambiguity of how the Wnt gradient is formed is that all vertebrate Wnts are post-translationally modified with a palmitoleate group, thus rendering them hydrophobic. As such, it is unlikely that the Wnt gradient is established simply through the diffusion of hydrophobic Wnts in the aqueous extracellular space. We have found that (1) cell-to-cell contact is necessary for WNT1 signaling, (2) that the co-expression of WNT1 with its intracellular transporter, Wntless (WLS), induces the formation of cellular projections, and (3) that WNT1 is localized within these projections. Furthermore, we have seen that the projections containing WNT1 are actin and not tubulin-based projections suggesting that these projections are filopodia. To test if filopodia are needed for Wnt signal transport we leveraged the ability of Myosin 10 to induce new filopodia and

found that Wnt signaling increased when we co-expressed Myosin 10, WNT1, and WLS in a co-culture assay. Similar to Myosin 10, other reports have shown that ROR2, a tyrosine kinase Wnt receptor, also induces filopodia growth when co-expressed with WNT8A, and was localized to WNT8A positive filopodia, thus suggesting that ROR2 may have a role in both Wnt producing and Wnt responding cells. Taken together, I hypothesize that overexpression of ROR2 in WNT1 producing cells will promote WNT1 signaling by enhancing the production of filopodia. To test this hypothesis, I first investigated the endogenous expression levels of ROR1 and ROR2 in HEK293T cells using RTPCR. Results show that HEK293T cells express endogenous ROR1 and ROR2. I then utilized a co-culture assay in which WNT1-producing HEK293T cells, transfected with WLS, were mixed with Wnt-responding HEK293T cells, which were transfected with a SuperTopFlash Wnt reporter. In order to test the role of ROR2 in Wnt-reception and Wnt-production, cells were transfected +/- ROR2. Though ROR2 is not typically linked to β-catenin dependent signaling, our results show that the overexpression of ROR2 in Wnt responding cells increase signaling. Furthermore, we show that ROR2 augments WNT1 signaling when present in WNT1 producing cells. These results are novel in that ROR2 not only plays a role in WNT1 reception, but also in WNT1 production. If our hypothesis is valid, we would expect that the ROR2 expressing HEK293T cells would be making more filopodia. We are currently testing this prediction.

P2537/B795

MicroRNA Regulation of Mesodermal Cell Fate in Early Development.

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MicroRNAs are short non-coding RNAs that fine-tune gene expression by pairing to the 3' untranslated region (3'UTR) of protein coding mRNAs to repress translation. These non-coding RNAs are vital for regulating early developmental processes. MicroRNA-124 (miR-124) is an evolutionarily conserved small RNA that has been examined in the context of neural specification; however, this research defines the novel role of miR-124 in the specification and development of two different mesodermally-derived immune cells: pigment and blastocoelar cells. Early in development, these two groups of immune cells share the same progenitor cell type and later must make a binary fate decision to differentiation. In the sea urchin, Nodal signaling activates genes expressed in blastocoelar cells and inhibits key transcription factors in pigment cell specification at about 10h into development. Two hours later, the Delta/Notch signaling pathway activates genes expressed in both blastoceolar and pigment cells. To examine the function of miR-124 in the early embryo, we microinjected the miR-124 inhibitor into newly fertilized sea urchin eggs. Results indicate that miR-124 inhibition caused ectopic misexpression of *Ese, GataC and Scl* which are transcription factors that controls the specification of blastocoelar cells. miR-124 knockdown (KD) led to increased number of blastcoelar cells at the expense of pigment cells. Based on our results, I hypothesize that miR-124 regulates both blastocoelar and pigment cell development by suppression of Nodal and Notch signaling pathways. We bioinformatically identified potential miR-124 binding sites within the 3'UTR of *Notch*, *Nodal* and their downstream targets *Gcm* and *Ese* in the pigment and blastoceolar cells, respectively. To test the direct regulation of miR-124 of *Notch*, we cloned the *Notch* 3'UTR downstream of *Renilla* luciferase reporter construct and identified one functional miR-124 regulatory site. The impact of miR-124's suppression of *Notch* was tested with miR-124 target protector (miR-124 *Notch* TP), which competitively blocked the miR-124 regulatory site within the *Notch* 3'UTR. Surprisingly, *Notch* TP resulted in increased expression of *GataC and Scl* of the blastocoelar cells, as well as increased expression of *Gcm and Pks* of the pigment cells. Interestingly, this increase of *Gcm* expression domain correlated with a doubling of differentiated pigment cells in the

larvae. Results from this study will identify the regulatory mechanism of miR-124 in mesodermal cell fate determination.

P2538/B796

Unraveling the Gene Regulatory Network of Early Lineage Commitment and Context-Dependent TGF-beta Response.

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During mammalian development, progenitor cells differentiate and commit to ever-more-specific lineages. The cell's response to signaling molecules changes with commitment: selecting a given lineage entails reinterpreting signals that would have previously promoted an alternative lineage. This process of committing to a lineage by changing response to signaling molecules is of critical importance for proper development, but the dynamics and mechanism that govern the process are unclear. We investigate the binary response of human embryonic stem cells to BMP and Activin a during differentiation toward the bipotent ectoderm lineage by discovering a two-gene reaction coordinate that allows us to monitor the dynamics of differentiation and lineage commitment in real time. We show that knowledge of the expression levels of OCT4:RFP and SOX2:YFP in a double-tagged reporter line is sufficient to predict the cell's commitment to bipotent ectoderm and therefore its loss of mesendoderm-competent response to TGF-beta signals; in contrast, classical neural/ectodermal marker genes activate too late to be useful for this purpose. We predict candidate genes of the commitment gene regulatory network (GRN) using detailed RNA-seq and ATAC-seq analysis of cells that are pre- and post-commitment to bipotent ectoderm. We are able to extend or shorten the window of mesendoderm competency by overexpression of the putative GRN components, thereby validating their role in the network. This study elucidates the GRN governing commitment and provides high-time-resolution details of the commitment process.

P2539/B797

Erk Signaling Dynamics Is Coordinated with Mitosis to Direct Epiblast and Primitive Endoderm Specification in Mammalian Blastocysts.

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During preimplantation development, mammalian embryos rely on constant crosstalk between signaling networks and gene expression to robustly specify the trophectoderm, epiblast (EPI), and primitive endoderm (PRE) lineages in the absence of maternal patterning cues. The latter two lineages emerge from a core of cells known as the inner cell mass (ICM) in a process dependent on ERK signaling downstream of fibroblast growth factor receptors (FGFRs). Higher levels of FGFR signaling promote PRE specification while inhibition of FGFRs or ERK promote EPI commitment. How these signaling differences arise during in vivo development remains unclear because it was previously impossible to study ERK activity in live embryos. To address this, we generated a mouse model that utilizes kinase translocation reporter (KTR) technology to monitor ERK activity in real time in live embryos cultured ex vivo. Single cell analysis of ERK KTR embryos revealed that all ICM cells exhibit prolonged periods of high ERK activity; however, a subset of ICM cells exhibit transient periods of ERK inactivity immediately following mitosis. Sister cell pairs adopt this low ERK activity status independent of their position within the ICM. Importantly, cells that exhibit low ERK activity ultimately express higher levels of NANOG, an EPI specific

marker, compared to cells that adopt high ERK activity. These data provide evidence for coordination of ERK activity with cell cycle progression in the ICM to direct EPI and PRE differentiation in developing blastocysts.

P2540/B798

Visualizing the Proliferation/differentiation Decision Using a CDK Activity Sensor during Metazoan Development.

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During organismal development, differential regulation of the cell cycle is critical to many cell biological processes, including cell fate specification and differentiation. While the mechanisms of the cell cycle are well studied, how the control of cell cycle is linked to differentiated cell behavior remains poorly understood. To truly understand this interplay, we must be able to directly and precisely measure cell cycle state. In order to characterize cell cycle state in living animals, we adapted a CDK2 biosensor for in *vivo* use. We generated transgenic strains in the nematode, *C. elegans*, and zebrafish, *D. rerio*, and performed transient RNA injections in the frog, *X. laevis*. The biosensor reports Cyclin-Dependent Kinase (**CDK**) activity as the cytoplasmic/nuclear localization ratio of a GFP-tagged human CDK2 substrate, DNA Helicase B (**DHB**). Phosphorylation of the sensor by CDKs causes its translocation from the nucleus to the cytoplasm. We have modified this sensor to allow for automated assessment of cell cycle state using a U-Net based automated image segmentation approach. Unlike other live cell cycle imaging tools (e.g., FUCCI), DHB can be used to distinguish actively cycling cells in the G1 phase of the cell cycle versus quiescent or terminally differentiated cells in G0. We have used this biosensor to quantify lineage specific differences between cycling cells, and to examine the link between quiescence and differentiation. We examine the interplay between proliferation, differentiation, and morphogenesis across multiple metazoan species and provide new biological insights into the control and timing of the metazoan cell cycle during specification and differentiation.

P2541/B799

An Increase in the Duration of an Extended G1 Period Enables Stochastic Competition between Terminal Cell Differentiation and Cell-cycle Re-entry.

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Terminal cell differentiation is essential for developing and maintaining tissues in all multi-cellular organisms and typically requires that proliferating progenitor cells exit the cell cycle. However, the relationship and timing between the underlying opposing processes, proliferation and terminal differentiation, is not yet understood. Using adipogenesis as a model system for terminal cell differentiation, we delineate the timing between cell-cycle exit and terminal differentiation by live-cell imaging of cell-cycle reporters and expression of PPARG, a master regulator of differentiation. We show that, during terminal cell differentiation, the levels of PPARG and of the CDK inhibitor p21 are coupled,

and both gradually increase after mitosis. Expression of p21 regulates the duration of a variable extended G1 phase that allows some cells sufficient time to reach a PPARG threshold for differentiation instead of entering the next cell cycle when PPARG is again suppressed. By way of regulating the duration of an extended G1 phase during terminal differentiation, progenitor cells can stochastically control the number of final cell divisions and thus, the total number of differentiated cells.

P2542/B800

Mating Yeast Cells Use an Intrinsic Polarity Site to Assemble a Pheromone-gradient Tracking Machine.

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The mating of budding yeast depends on chemotropism, a fundamental cellular process. The two yeast mating types secrete a peptide pheromone that binds to GPCRs on cells of the opposite type. Cells find and contact a partner by determining the direction of the pheromone source and polarizing their growth toward it. Actin-directed secretion to the chemotropic growth site (CS) generates a mating projection. When pheromone-stimulated cells are unable to sense a gradient, they form mating projections where they would have budded in the next cell cycle, at a position called the default polarity site (DS). Numerous models have been proposed to explain yeast gradient sensing, but none address how cells reliably switch from the intrinsically determined DS to the gradient-aligned CS, despite a weak spatial signal. Here we demonstrate that, in mating cells, the initially uniform receptor and G protein first polarize to the DS, then redistribute along the plasma membrane until they reach the CS. Our data indicate that signaling, polarity, and trafficking proteins localize to the DS during assembly of what we call the gradient tracking machine (GTM). Differential activation of the receptor triggers feedback mechanisms that bias exocytosis upgradient and endocytosis downgradient, thus enabling redistribution of the GTM toward the pheromone source. The GTM stabilizes when the receptor peak centers at the CS and the endocytic machinery surrounds it. A computational model simulates GTM tracking and stabilization, and correctly predicts that its assembly at a single site contributes to mating fidelity.

P2543/B801

Elucidating the Role of Tpc2 in *Dictyostelium* Cell Proliferation and Development.

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Dictyostelium discoideum is a preferable multicellular model system for the study of various cellular and molecular mechanism. Starvation induced multicellular development is achieved by cells aggregation and culmination to form a fruiting body having two terminally differentiated cell types: prestalk and prespore cells. During differentiation prestalk cells undergoes autophagy leading to autophagic cell death (ACD). Calcium plays an important role in the process of cell differentiation and high ca^{2+} load shows biasness towards prestalk pathway that involve autophagic cell death in *Dictyostelium discoideum*, Therefore we selected Two-pore calcium channel 2 (TPC2) for our studies to analyze its role in cell proliferation, development and autophagy in *Dictyostelium*. Two pore channels (TPCs) are the members of voltage-gated ion channel superfamily consisting of two subunits of six transmembrane domains that are ubiquitously present in organelles of animals and plants. Two strains of TPC2 [overexpressor ($TPC2^{OE}$) and knockout ($TPC2^{KO}$)] were created. Role of TPC2 in growth, development and cell death was analyzed. Involvement of TPC2 in autophagy fluxes was measured by co-expression of TPC2 mutant strains with RFP-GFP-ATG8 (autophagy marker) and with GFP-Tkt1 (cytosolic marker). Here we report the *spatio-temporal* mRNA expression patterns of TPC2 in *Dictyostelium*. We observed that

the mRNA transcript of TPC2 was present throughout the growth and developmental stages of Dictyostelium with highest expression during later stages of development i.e. slug and culminant. We also observed that the absence of TPC2, increases the cell proliferation rates and delays development of the *Dictyostelium*. Our results showed that TPC2 is a calcium channel which is involved in growth, development and has a significant role in autophagy.

P2544/B802

Cilia and Centrioles Are Extruded during Multiciliated Cell Transdifferentiation in Xenopus Embryonic Skin.

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While there have been significant advances in how a cell commits to a certain cell fate, the mechanisms that govern cell fate malleability after this occurs remains relatively unknown. Transdifferentiation is a process whereby a differentiated cell can directly transform into another differentiated cell type without regressing through a proliferative, stem cell-like state. It has been proposed that multiciliated cells (MCCs) are capable of undergoing transdifferentiation. The skin of Xenopus embryos contains numerous MCCs that work to create a directed fluid flow over the epithelial surface. To generate this flow these cells become extremely specialized, containing approximately 150 evenly spaced motile cilia each anchored to a basal body and connected via an extensive cytoskeletal network. Additionally, these cells have massive numbers of mitochondria that provide the energetic requirements to fuel cilia motility. The extreme specializations of these cells provide a unique opportunity to understand the steps required to undergo transdifferentiation. We have found that as embryos continue to mature the cilia-induced flow decreases until it ultimately disappears. Interestingly, during this window of development, by utilizing biochemical approaches and long-term live cell imaging with a light sheet microscope, we have observed cilia cleavage and extrusion of centrioles from the surface of MCCs as they begin the process of transdifferentiation, each representing a distinct step. We found that cilia are first cleaved off of the surface of MCCs. Then, basal bodies are collected and removed from cells in a dynamic and rapid manner where cells pinch off and extrude a large basal body-filled vesicle. We hypothesize that the elimination of these structures is a prerequisite to continue transdifferentiation. Moreover, the treatment of Xenopus embryos with a gamma-secretase inhibitor can delay this transdifferentiation process suggesting that Notch signaling likely plays a role in cell malleability. By combining our live imaging approaches with MCC lineage tracing studies we will be able to better understand additional signaling pathways that govern transdifferentiation. Ultimately, this will be useful to identify the steps required for a cell to undergo transdifferentiation and alter its cellular identity.

P2545/B803

Genetic Fate Mapping Reveals Heptatoblast Potential and Lineage Relationships between Endoderm-derived Organs in the Mouse.

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The Definitive Endoderm (DE) is the embryonic germ layer that will produce the bulk of the gastro-intestinal tract as well as the associated organs including liver, lungs and pancreas. The focus of our laboratory is to understand how the DE initiates liver formation. Hepatoblast are the first liver cell type produced by the embryo and this population will produce the two mature liver cell types: hepatocytes,

the cells that produce bile, and cholangiocytes, the cells that form the bile ducts. We recently used a genetic inducible fate mapping (GIFM) system to demonstrate that single hepatoblasts were multi-potent, producing hepatocytes and cholangiocytes. An intriguing observation was that a percentage of the hepatoblasts also appeared to produce endothelial cells, the cells that form the vasculature and are typically believed to derive from the mesoderm, a separate germ layer. To learn more about how hepatoblasts furnish the liver, we used a similar GIFM as used previously and labeled cells with a Cre-inducible reporter (Rosa26LacZ) paired with a DE-expressing Cre-ER transgene (FoxA2CreER). We identified that a low dose of tamoxifen that result in infrequently labeled descendants that can be detected using X-Gal. We used immunohistochemistry with the endothelial marker, ERG1, to identify the presence of labeled endothelial cells in all 5 livers that contained LacZ positive cells at E12.5. We propose to use the GIFM strategy to determine when hepatoblasts have this potency by examining embryos at earlier time-points starting with E10.5. Together these results will provide further evidence for the provocative hypothesis that DE cells can produce cell-types previously believed to be formed by the mesoderm and may help to explain the remarkable regenerative capacity that is unique to the liver.

P2546/B804

Transcriptomic an alysis of the Compensatory Response to Notch Signaling Perturbation during Embryonic Development.

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During early development, embryos display a remarkable degree of plasticity and resilience. They can redirect cellular movement, proliferation, and even fates of determination in order to compensate for physical, chemical, and genetic perturbations. At some point in the developmental process, however, this plasticity diminishes, and as they grow into mature organisms, embryos can no longer recover from significant damage without scarring or defects. Understanding the molecular basis of embryonic plasticity has applications in cancer therapy and regenerative medicine. Therefore, we sought to investigate how developing *Xenopus* embryos respond to and compensate for perturbations to the Notch signaling pathway; the Notch signaling pathway is a conserved juxtacrine signaling network that is responsible for directing early neurogenesis during vertebrate development, and whose dysregulation has been linked to various types of neurodegenerative diseases and cancers. Initial experiments have suggested that *Xenopus* embryos are able to elicit a remarkable response to both over- and underexpression of this critical pathway; injection of Notch mRNA constructs that either over- or underexpress the pathway results in initial perturbation to the system as assayed by in *situ hybridization* of the pan-neural marker gene *tubb2b*, but marker gene expression as well as normal stimuli responses are restored in embryos by early tailbud stages of development. In order to understand the molecular basis of this plasticity, we have begun to characterize this compensatory response by performing dosage experiments and RNA-sequencing for Notch over- and underexpression at early neurula, tailbud, and tadpole stages. RNA-Seq analysis suggests that this response includes regulation of both the Notch and Wnt signaling pathways as well as changes in cell proliferation, apoptosis, and calcium activity. Further, it also suggests that while embryos appear anatomically healthy by tailbud stages, at the gene expression level, the compensatory response lasts well into tadpole stages. Additionally, preliminary data suggests that the degree of recovery is dosage-dependent. Finally, having established that *Xenopus laevis* embryos compensate in response to perturbations in the Notch signaling pathway, we have asked

whether this response is conserved across vertebrate species. We have begun characterizing the compensatory response to Notch signaling perturbation in two closely related species, the tetraploid *Xenopus borealis* and diploid *Xenopus tropicalis*. The findings from these experiments will yield insight into both the question of how conserved the response to Notch signaling perturbation is in closely related species, as well as whether ploidy is involved in this response.

P2547/B805

Mechanical Control of Mucociliary Epithelia Regeneration on the Surface of Embryonic Aggregates.

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Mucociliary epithelia line organs throughout the body including lung, gut, and kidney and play a key role in maintaining the protective mucosal barrier against foreign particles. This first line of shield often disrupted through injury, surgery, and diseases and require a regeneration to restore its structure and function. Here we introduce a mucociliary epithelia organoid that rapidly recapitulate the regeneration process of epithelialization and cell specification on the surface of the embryonic aggregate. Following disruption of embryonic tissue architecture and assembly of a compact aggregate using embryonic mesenchymal cells, regeneration first proceed with restoration of an epithelium, transitioning from mesenchymal cells at the surface of the aggregate. Surface cells selectively establish apico-basal polarity within 5 hours and differentiate into mucus-secreting goblet cells that complete with muticiliated cells within 24 hours. Regeneration coincides with nuclear translocation of the putative mechanotransducer YAP1 and a sharp increase in aggregate stiffness. Acute and chronic alterations of tissue stiffness reveal that tissue mechanics drives the regeneration of a mucociliary epithelia on the surface of mesenchymal aggregates.

P2548/B806

Mutations in Vacuolar-type H+-atpase (v-atpase) Cause Necrosis-like Cell Death of Mechanosensory Hair Cells.

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The vacuolar type H^+-ATPase (V-ATPase) is a membrane-bound, multi-subunit complex that pumps protons from cytoplasm into organelle lumens or into extracellular space. V-ATPase activity is important for regulating pH of cellular compartments, and hence has been implicated in multiple cellular processes. Previous genetic analyses indicate V-ATPase has vital functions during embryonic development. However, mechanisms by which V-ATPase impacts cellular behaviors during development remain poorly understood. Here, we show mutations in two core V-ATPase subunits—*atp6v1f* and *atp6v1h* that are required for V-ATPase function—disrupt neuromasts in the lateral line of the zebrafish embryo. Neuromasts are mechanosensitive organs that are used by aquatic vertebrates to detect vibrations. Neuromasts consist of a cluster of non-sensory support cells surrounding centrally located sensory hair cells that are functionally identical to mammalian inner ear hair cells. Interestingly, V-ATPase mutations have been identified in patients with sensorineural deafness, but underlying mechanisms are unclear. In *atp6v1f*$^{-/-}$ and *atp6v1h*$^{-/-}$ mutant zebrafish embryos, we observed smaller and misshapen neuromasts. These neuromasts had a reduced number of sensory hair cells, but support cell numbers were normal. V-ATPase mutant neuromasts also showed the presence of pyknotic nuclei that increased over time. Using molecular markers, we found that loss of V-ATPase specifically reduces the

survival of hair cells, but not support cells. Previous work in cell cultures and zebrafish embryos indicates loss of V-ATPase function can induce Caspase 3-dependent apoptotic cell death in specific cell types. Surprisingly, we found that V-ATPase loss of function induced Caspase 3-independent death in hair cells undergoing functional maturation. In addition, Caspase 8 levels were similar in mutant and wild-type hair cells, which is consistent with a non-apoptotic mechanism. Confocal imaging of living embryos indicated mutant hair cells undergo a necrosis-like mode of cell death: mutant hair cells were found to swell and then rupture, rather than form apoptotic blebs characteristic of apoptotic cell death. These findings reveal a new function for V-ATPase in hair cell survival, where loss of V-ATPase induces a Caspase-independent and necrosis-like cell death pathway. This may have implications in furthering our understanding of the causes of sensorineural deafness.

P2549/B807

Evolution of an imal Cell Fate Determination.

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The origin of animal multicellularity coincided with the evolution of mechanisms for determining the identities and fates of different cell types. Remarkably, the closest living relatives of animals, the choanoflagellates, uniquely share with animals regulatory genes that influence cell fate in animal development. Here, we use ATAC-seq and RNA-seq to profile gene regulatory changes between four different life history stages in the model choanoflagellate *S. rosetta*. In these data, we identify RNA-binding proteins with a universal role for germ cell determination in animals that may regulate gametogenesis and the transition from a benthic to pelagic lifestyle. These results will help illuminate the evolution of cell fate determination pathways, especially a potential core regulatory program that gave rise to the animal germ line.

P2550/B808

Cell Fusion Induces a Structural-to-transcriptional Differentiation Switch through Membrane Remodeling, Transient Energy Deprivation and Yap1 Cytoplasmic Retention.

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Developmentally-regulated cell fusion generates highly differentiated skeletal muscle, bone and placental cells, but whether the act of fusion contributes to differentiation, independent from tissue-specific cues, is unclear. To address this, we employed a viral-protein mediated cell fusion model involving undifferentiated cells in tissue culture. RNA-sequence analysis revealed that cell-cycle arrest after fusion was accompanied by a dramatic shift from proliferation-associated to differentiation-causing transcripts. This shift coincided with nuclear depletion of the proliferation-promoting factor YAP1 triggered by enhanced endocytosis of glucose transporters, and transient decrease of cytoplasmic glucose and ATP levels. This low energy state stimulated AMPK activation promoting YAP1 inhibition. Impairing either endocytosis or AMPK prevented nuclear depletion of YAP1 and cell-cycle arrest after cell fusion. Together, these data show that structural changes and their effect on cell-bioenergetics can induce a cell differentiated-like state in response to cell fusion. This occurs in the absence of tissue-specific signaling molecules.

Germ Cells, Gametogenesis, and Fertilization

P2551/B809

Ras Inhibition of Contractile Ring Function in *Drosophila* Primordial Germ Cells.

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While Ras has been studied extensively for its role in cancer and tumorigenesis, there is still much unknown about how its activity is toggled and modulated during normal development and morphogenesis. We can use the formation of primordial germ cells (PGCs) as a model system for understanding transcription-independent regulation of cellular morphogenesis through Ras. PGCs are the first cells to form in the syncytial Drosophila embryo, separated from the rest of the embryo by a spindle-independent cleavage that constricts pole buds. This process requires a ubiquitin ligase adaptor Germ-cell-less (GCL), which has been shown to degrade Torso (Tor), a receptor tyrosine kinase. Tor activates Ras signaling, and without suppression of this activation, PGCs will not form. Later in embryogenesis, Ras is known to initiate the Raf/MEK/MAPK signaling cascade, leading to the transcriptional upregulation of somatic terminal genes. However, at the time of PGC formation, the embryo still has not activated zygotic transcription, and relies entirely on the maternal contribution of RNAs and proteins. Therefore, Ras acts in a transcription-independent way to antagonize the formation of PGCs. Our data suggest that Ras is activating RalA, an effector of somatic cellularization, thus inhibiting pole bud constriction from occurring.

P2552/B810

A Demographic Model to Estimate the Cell Division Rates of Stem Cells and Transit-amplifying Cells.

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Regulation of adult stem cell division and differentiation is critical for maintenance of tissue. In many tissues, the stem cell progenitors proliferate for several rounds, termed as transit amplification (TA) divisions, prior to terminal differentiation. Stem cells are described as slow-cycling cells which retain their mitotic potential for a long duration, whereas their transit-amplifying daughters proliferate relatively rapidly and have limited mitotic potential. We used *Drosophila* spermatogenesis as a model system to study the regulation of stem cell and TA cell mitosis. We describe a demographic model which uses the theory of probability to predict the lifetimes of the germline stem cells and TA stages at steady-state in the adult testis using fixed-tissue data. Numerical analysis of the data derived from cyst distribution, mitotic indices and the germ-cell death counts along with the time-lapse imaging of the germline stem cell (GSC) division in wild-type background suggested that halfway into the germline TA, the cell division periods are reduced by about 2-folds. We verified the model by knockdown of known cell cycle regulators such as CycE and CDK1 and observed an expected slowdown of the GSC and TA divisions. Upon overexpression of String, we noted a speed-up of the early germline divisions, as expected. Using the same model, we then investigated the role of somatic EGFR signaling in the regulation of GSC and TA cell division rates. Previous reports suggested that high levels of somatic EGFR signaling could induce premature germ cell differentiation. We found that localized gain of somatic EGFR signaling by Spitz (EGF ligand) overexpression or knockdown of a feedback molecule Argos led to a slowdown of the GSC and TA divisions, suggesting a dose-dependence between the somatic EGFR signaling and the rates of germline divisions. Together, these results established the efficacy of our

demographic model in estimating the role of different intrinsic and extrinsic components in the regulation of GSC and TA divisions.

P2553/B811

Adipocyte Nutrient Sensing Controls the Ovarian Germline Stem Cell Lineage in *Drosophila Melanogaster*.

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External factors, particularly nutritional status, can positively or negatively impact reproduction by affecting gamete production. The stem cell-supported ovary in *Drosophila melanogaster* sustains robust reproductive capacity of adult females. Female flies fed a protein-poor diet lay significantly fewer eggs than those fed a protein-rich diet. This response to diet is mediated by the activity of nutrient-sensing pathways within the ovary, which control germline stem cells (GSCs) and their progeny. It is well-established that target of rapamycin (TOR)-mediated and insulin/insulin-like growth factor signaling within the ovary control GSC maintenance and proliferation, germline survival, vitellogenesis and ovulation. Our previous studies revealed that inter-organ communication serves as an added level of control in modulating the ovarian response to changes in diet. More specifically, distinct nutrient sensing pathways function within adipocytes, the major cellular component of *Drosophila* fat tissue, to remotely control cellular activity in the ovary. We find that insulin signaling within adipocytes influences GSC maintenance as well as germ cell survival using distinct downstream signaling effectors. We have also shown that amino acid sensing by adipocytes controls GSC maintenance and ovulation of mature oocytes via activity of the amino acid response pathway and TOR, respectively. Currently, we are using genetic and cell biological tools to determine how remote nutrient sensing modulates oocyte development by 1) deciphering the complex molecular mechanisms of insulin signaling within adipocytes and 2) assessing the contribution of the two downstream effects of adipocyte amino acid response pathway activity. Ultimately, our goal is to identify adipocyte factors downstream of insulin signaling and amino acid response pathway activation that modulate GSCs and other cell types in the *Drosophila* ovary. This work will illuminate how cell-cell communication between organs regulates the germline to match an organism's nutritional status to an investment in egg production.

P2554/B812

Levels of the Adaptor Protein, Dreadlocks, Must Be Precisely Regulated during Development of the *Drosophila* Egg Chamber.

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Intercellular communication is critical for the function of any multicellular organism; many medical conditions can be attributed to errors in cellular connections such as heart disease, neurological disorders, and infertility. Cellular communication is especially important during gamete formation, as it often relies on intercellular bridges to connect developing germ cells to each other or to supporting cells. An excellent system to study intercellular bridges is the germline of the developing *Drosophila* egg. Mature eggs are derived from multicellular structures called egg chambers; each egg chamber contains a germ cell cluster (one oocyte and fifteen nurse cells), which is surrounded by a layer of somatic cells. The germ cells are connected to each other through intercellular bridges, called ring canals, which form following incomplete cytokinesis and expand 20-fold in diameter during oogenesis. The stability and regulated expansion of the ring canals are essential for fertility. We have identified a novel role for the

conserved adaptor protein, Dreadlocks (Dock), in regulating ring canal size and germ cell stability. Dock localizes to the germline ring canals, and depletion of Dock leads to a modest, yet significant, increase in ring canal diameter. Over-expression of Dock in the germline leads to dramatic defects in ring canal structure, nurse cell fusion, and apoptosis. We have utilized a series of UAS-HA-Dock constructs with point mutations in each of the four domains (SH3-1, SH3-2, SH3-3, SH2, and SH3-1,2,3) in order to determine which domains are required for Dock localization and for the over-expression phenotype. Future experiments will identify the specific Dock-interacting proteins that regulate ring canal structure and membrane integrity.

P2555/B813

Examining Size Scaling Relationships in the Developing *Drosophila* Egg Chamber.

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Proper regulation of the size of cellular structures is essential for the normal function of cells, and a scaling relationship is often seen between cell size and the size of intracellular structures and organelles. For instance, larger cells often have larger structures or more organelles than smaller cells. Although these scaling relationships have been observed, few studies have been done to compare the scaling of cellular structures within and between species, especially in growing tissues. We are using the developing fruit fly egg chamber as a model system to study size scaling relationships throughout development. The developing egg chamber is composed of a central cluster of germ cells (fifteen nurse cells and one oocyte) that are connected to each other through intercellular bridges called ring canals; this germ cell cluster is surrounded by a layer of somatic cells. During oogenesis, the egg chamber undergoes a 20-fold increase in volume, and many of the structures must also undergo significant growth to support the formation of a viable egg. We have chosen to initially focus on the size scaling of two structures: the germline intercellular bridges, or ring canals, which connect the nurse cells to each other and to the oocyte, and the nurse cell nuclei. We have found that both nuclear size and ring canal size scale with egg chamber size across multiple *Drosophila* species. Similar analysis of a collection of *D. melanogaster* lines that have been artificially selected to produce either big eggs or small eggs suggests that this size scaling relationship is also maintained when looking within a single species. Future studies will follow up on these data and explore the mechanisms that could establish and maintain these size scaling relationships during tissue growth.

P2556/B814

Kar4 Is Required at Multiple Stages during Meiosis and Engages in a Function Specific Interaction with a Novel Meiotic Regulator, Kfc1.

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Budding yeast can embark on several distinct differentiation pathways including mating and meiosis. Although these pathways are vastly different in induction and outcome, both require the highly conserved eukaryotic protein Kar4. Kar4's mating function involves interacting with the transcription factor Ste12 to activate the expression of genes required for nuclear fusion. Our understanding of Kar4's meiotic function is much less complete. The mammalian ortholog of Kar4, Mettl14, is a key member of the WTAP complex that catalyzes RNA methylation, but Kar4 is not required for RNA methylation in yeast. How then does it regulate meiosis? a "separation of function" mutation screen identified *KAR4*

alleles specific to either the mating or meiotic function. Surprisingly, the meiotic mutants fell into two classes revealing that Kar4 has two distinct meiotic functions. The defect associated with the first function (Mei) is suppressed by over-expression of the master meiotic transcription factor Ime1. Suppression of the second function (Spo) requires the over-expression of an additional gene, *RIM4*, encoding a translational regulator. These findings led us to hypothesize that the Mei function acts at the level of transcriptional regulation, whereas the Spo function acts at the level of translational regulation. How is Kar4 able to have three distinct functions? We hypothesized that Kar4 might have different function-specific binding partners (e.g. Ste12 during mating), which we sought to identify using immunoprecipitation coupled with mass spectrometry. We chose to focus on an uncharacterized interacting protein, Ygl036, which we have since named Kar4 Collaborator 1 (KFC1). Here, we confirm that Kar4 interacts with Kfc1 and show that the interaction is specific to Kar4's Spo function. *KFC1* is essential for meiosis and we have begun to characterize the *kfc1*Δ/Δ meiotic defect at both the cellular and molecular level.

P2557/B815

Understanding Translation Regulation during the Developmental Process of Meiosis.

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Meiosis is the developmental program where precursor diploid cells divide into non identical haploid gametes. Meiosis is conserved from yeast to humans, and budding yeast can be induced to synchronously undergo meiosis in the lab. Our lab utilizes budding yeast to study the diverse cellular reprogramming events that allow this process to occur through both gene regulation and organelle biology. My studies have focused on trying to understand how strong and diverse changes to the yeast translatome are modulated during meiosis, and what the function of these changes may be.

P2558/B816

FBF-1 and FBF-2 Coordinatedly Regulate Germline Stem and Progenitor Cell Proliferation and Differentiation in *Caenorhabditis Elegans*.

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Control of the balance of stem cell proliferation and differentiation is one of the central questions of stem cell biology relevant for our understanding of ageing, cancer and various degenerative disorders. Here, we report that FBF-1 and FBF-2, two Pumilio and FBF (PUF) family RNA-binding proteins in *C. elegans*, coordinately regulate the rate of both proliferation and differentiation of germline stem and progenitor cells (SPCs). The two FBF homologs share most of their target mRNAs (Kershner et al., 2010, Prasad et al., 2016 and Porter et al., 2018) and function redundantly in maintaining germline SPCs (Crittenden et al., 2002). Surprisingly, we found that FBFs have distinct effects on proliferation and differentiation of germline SPCs. The analysis of cell cycle dynamics of germline SPCs in different genetic backgrounds revealed that loss of *fbf-1* gene (*fbf-1(lf))* causes faster cell cycle rate of SPCs than the wild type, suggesting that FBF-1 protein causes slower SPC proliferation. By contrast, *fbf-2(lf)* causes slower progression through the G2 phase of the SPC cell cycle compared to the wild type, suggesting that FBF-2 facilitates progression through G2. Furthermore, estimating the rate of meiotic entry or differentiation in each *fbf* mutant compared to the wild type suggests that FBF-1 protein causes slower differentiation rate while FBF-2 promotes faster differentiation. Together, these data suggest that FBFs coordinatedly regulate cell proliferation and differentiation rates of germline SPCs. Quantitative PCR analysis of steady

state mRNA levels of FBF targets regulating proliferation and differentiation revealed that compared to the wild type, *fbf-1(lf)* leads to increased abundance of tested target mRNAs while *fbf-2(lf)* causes decreased abundance of the same mRNAs, suggesting that FBF-1 destabilizes target mRNAs while FBF-2 promotes accumulation of target mRNAs. We propose that the balance of FBF-1 and FBF-2 activities determines the steady-state abundance of target mRNAs and ultimately sets the rates of both cell cycle and meiotic entry in SPCs. Finally, we found that FBF-1 activity in SPCs requires CCR4-NOT deadenylase machinery and FBF-1 promotes deadenylation of its target mRNA. By contrast, FBF-2 activity in stem cells shows less dependence on CCR4-NOT, which reflects its amino acid sequence divergence from FBF-1. In conclusion, our study suggests a new mechanism for control of stem cell proliferation in concert with differentiation by PUF proteins through differential cooperation with CCR4-NOT deadenylase machinery, which may be conserved across species.

P2559/B817

Lipid Droplet-associated Proteins Are Required to Build the Eggshell Permeability Barrier In*C. Elegans*.

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The *C. elegans* embryo is surrounded by a rigid and impermeable eggshell that provides protection during early development. The eggshell contains four distinct layers that are built through the rapid secretion and deposition of extracellular glycoproteins and lipids. The innermost layer of the eggshell, called the permeability barrier, is formed during meiosis II via assembly of an uncharacterized class of lipids (likely to contain polyunsaturated fatty acids). Not only does the identity of permeability barrier lipids remain a mystery, it is also unknown what organelle synthesizes the lipids or how they are delivered to the extracellular environment. We previously conducted an RNAi screen to identify proteins involved in eggshell formation, and found that a number of lipid droplet-associated proteins were required to maintain the impermeability of the eggshell. Depletion of Seipin, Lipin, and Diacylglycerol acyltransferase (DGAT)-related protein led to disruption of the permeability barrier, with little effect on the assembly of other eggshell layers. Depleted embryos also contained enlarged spherical structures reminiscent of lipid droplets. These three proteins are known to regulate lipid droplet formation and function in diverse animal species, from worms to humans. Likewise, the uncharacterized protein PERM-1, which contains predicted hydroxysteroid epimerase and reductase domains, exhibited a similar permeable eggshell phenotype. GFP- and mCherry-tagged versions of the proteins co-localized with the lipid droplet marker perilipin-1 (GFP::PLIN-1) or the endoplasmic reticulum marker SP12::mCherry. We therefore hypothesize that lipid droplets are the site of synthesis and/or storage of lipids that are used to assemble the eggshell permeability barrier. Current studies are underway to examine lipid droplet dynamics when the permeability barrier forms at meiosis II, and to identify the relevant lipid species through lipidomics analysis.

P2560/B818

The Role of ZK813 Proteins in Sperm-egg Recognition and Eggshell Formation in C. Elegans.

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In metazoans, oocytes are covered by a layer of extracellular matrix that aids in fertilization and egg coat assembly. In the nematode *C. elegans*, the outermost layer of the egg coat is called the vitelline layer. Our lab previously characterized the first set of vitelline layer components - CBD-1, PERM-2, and PERM-4 - which form a protein complex on the nascent vitelline layer. *To* identify additional proteins involved in

vitelline layer assembly, we performed a co-immunoprecipitation of PERM-2 followed by mass spectrophotometry and identified the novel interactors ZK813.1 and ZK813.3, which are components of a four-gene cluster of paralogs that may have arisen due to a chromosomal duplication event. The ZK813 gene cluster was knocked out using CRISPR, and the worms were found to have a fertilization defect. We recently knocked out each individual ZK813 gene in the cluster to determine which one(s) are responsible for the phenotype. Additionally, we generated GFP and mCherry tagged versions of ZK813.1 and ZK813.3, respectively, using CRISPR. Both proteins were found in the spermatheca, and ZK813.3 also localized to the eggshell after fertilization. We are currently performing experiments to explore the structural relationships among PERM-2, PERM-4, CBD-1, and the ZK813 proteins. We are also conducting ZK813 co-immunoprecipitations to identify additional interactors.

P2561/B819

The Species-limited Membrane Protein; *Minus* Adhesion Receptor 1 (Mar1), Controls Gamete Membrane Interactions Immediately Preceding Fusion during Fertilization in *Chlamydomonas*.

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The essential first steps in the creation of a new, genetically distinct, individual diploid cell during fertilization are gamete recognition, membrane adhesion, and the fusion of the gamete lipid bilayers. In many eukaryotes, the ancient, conserved fusogen HAP2 is necessary for the gamete membrane fusion step during fertilization. The tight association of the two gamete membranes is a prerequisite for bilayer merger by HAP2, yet a protein pair controlling this adhesive event has yet to described. Here, we report the identification of a species-limited membrane protein, *Minus* Adhesion Receptor 1 (MAR1), on the *minus* gametes of the model green alga *Chlamydomonas reinhardtii,* that interacts during fertilization with the previously identified, species-limited *plus* gamete membrane adhesion protein, FUS1. MAR1 is expressed predominantly by *minus* gametes, and localized to the *minus* mating structure, which is the site of fusion with the cognate, FUS1-expressing mating structure of *plus* gametes. FUS1 from *plus* gametes and MAR1 from *minus* gametes associate with each other in *vivo* and recombinant forms of the proteins bind to each other in *vitro*, demonstrating a direct interaction. Disruption of the MAR1 gene in *minus* gametes completely blocks mating structure adhesion and gamete fusion, a phenotype corresponding to that of *fus1 plus* mutants. *Plus* gametes bearing the disrupted MAR1 gene are fully capable of mating structure adhesion and gamete fusion. Transformation of the MAR1 deletion *minus* gametes with a FLAG-tagged MAR1 gene rescues the ability of these mutants to fuse with *plus* gametes. Immunoprecipitation studies also indicate that endogenously-expressed MAR1 and HAP2, which are both localized on the *minus* mating structure, are biochemically associated with one another. Moreover, both degrade rapidly during a block to polygamy after gamete fusion. These initial studies of the MAR1-FUS1 interaction in *Chlamydomonas* fertilization suggest the model that during the gamete membrane fusion reaction, species-limited membrane proteins bring the plasma membranes of gametes into close proximity, thereby allowing the conserved eukaryotic class II fusion protein HAP2 to perform its function of bilayer merger. Thus, while the introduction of the ancient fusogen HAP2 was likely the key factor responsible for the origin of two-parent sexual reproduction in eukaryotes, the gamete membrane adhesion proteins could theoretically have served as the initial incompatibility factors responsible for the origin of species.

P2562/B820

Proteome-wide Phosphorylation Dynamics during Oocyte Arrest, Meiosis, and the Transition to Embryogenesis.

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Cell cycle transitions throughout fertility and development depend on the robust control of the cell division apparatus across changing physiological contexts. Oocytes exist in an extended primary cell cycle arrest, persisting for up to decades in humans. After hormonal stimulus, oocytes execute two highly distinct and specialized divisions during the process of meiosis, undergo fertilization, and then transition to embryonic mitoses. Our goal is to understand how the cell division machinery is modulated during this dramatic developmental transition from prophase I arrest through early embryogenesis. Using the sea star *Patiria miniata*, which displays a high synchrony of meiotic events, we performed quantitative proteomics on oocytes at intervals from the prophase I arrest through the first embryonic cleavage. Despite the substantial rearrangements to the cell division machinery during this window, we find that the proteome is remarkably stable, with very little changes in protein levels. In contrast, we find the protein phosphorylation landscape is highly dynamic with multiple distinct waves and patterns of phosphorylation. We find that the prophase I arrest is defined by a uniquely low phosphorylation state achieved through high phosphatase activity. Indeed, we demonstrate that PP1/PP2A phosphatase activity is essential to maintain the prophase I arrest. Upon meiotic resumption, we identify waves of phosphorylation both unique and common to the meiotic divisions and first embryonic cleavage. In particular, our work identifies the behaviors of key regulatory kinases and phosphatases throughout the oocyte-to-embryo transition, as well as phosphorylation dynamics of their putative substrates. Our results provide a rich dataset of phosphorylation events that drive the rapid rearrangements to the cell division apparatus during the oocyte-to-embryo transition.

P2563/B821

Effect of Natural Extracts on Viability of Sea Urchin Eggs.

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Sea urchins, like humans, are deutrostomes and they share many common genes with higher order mammals. This makes them an ideal organism for embryonic development studies. However, harvested sea urchin eggs become heavily infested with a certain species of protist and disintegrate within 24 hours. The study investigated the effect of plant extracts of *Nigella sativa* and *Moringa oleifera* on the viability of sea urchin eggs. Freshly spawned eggs from the sea urchin *Lytechinus variegatus* were incubated with water extracts of both plants in a 1:1 ratio. Intact eggs were observed and counted on a hemocytometer for six days. Results indicate that while *Nigella sativa* extracts did not prevent the disintegration of eggs, phytochemicals of *Moringa oleifera* were able to preserve many egg cells for six days in intact conditions, far longer than the control. Future studies will focus on identifying and isolating the phytochemicals responsible for the protective effects of Moringa oleifera.

P2564/B822

The Effects of Triclosan on the Development of *Lytechinus* Variegatus Embryos.

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This project aims at investigating the effects of triclosan on the development of the sea urchin *Lytechinus variegatus* embryos. Gametes of *L. variegatus* were extracted from spawned adult urchins using potassium chloride (KCl) and in *vitro* fertilization was carefully performed. Fertilized gametes were treated with triclosan at nominal concentrations (0.5, 1, 5, 10 and 20 µM) allowing for embryogenesis to take place. Viability and development of embryos were analyzed using a light microscope. Embryos were counted using a hand drawn grid. Results indicate that embryos exposed to low triclosan concentration (0.5-1 µM) displayed normal development until the pluteus stage, while embryos exposed to high triclosan concentrations (>5 µM) displayed low viability and arrested development at early stages of embryogenesis. Results of this project suggest that higher concentrations of triclosan might have potential deleterious effects on embryonic development of *L. variegatus.* Future results will focus on studying the effect of triclosan on the rate of fertilization and the possible reason for the harmful effects of Triclosan.

P2565/B823

Protein an alysis of Infertile Male Testis by Two-dimensional Electrophoresis-relationship between Johnsen's Score and Protein Spots-.

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Currently, approximately 15 % of couples who want children are sterile and about half of them are attributed to male infertility. The causes of male infertility might include protein or chromosomal abnormalities due to genetic mutations, stress, lifestyle related disease such as obesity or smoking, aging and spermatogenic dysfunction such as azoospermia. In this study, we focused on proteins and aimed to find the relationship between the disease stage (Johnsen ’s score: JS) and protein spots by two-dimensional electrophoresis. JS has been used for sperm diagnosis, which evaluate male infertility, and expresses 1-10 scores at which stage the differentiation has been abrogated in spermatogenic process. The higher the score, the closer to normal differentiation. The testicular specimen of each JS was used as a sample for protein analysis. After protein extraction, protein spots were detected by two-dimensional electrophoresis (isoelectric focusing:pI 3-11, 19 hours, SDS-PAGE:4-20%, 180 V, 35 minutes) and silver staining. Samples with JS 7-10 were used as control. Twenty protein spots specific for sterile patients could be found after comparing samples derived from normal and sterile patients. In addition, many spots were lost in sample derived from lower score JS patients. Two protein spots defective in 1-4 JS scores were supposed to be involved in the arrest of differentiation after the spermatocytes, and suggesting that a protein spot only expressed in the control might be necessary for the completion of spermatogenesis. After analysis of more samples per each JS stages, it would be possible to correlate individual spots to each JS stages and spermatogenic process. Furthermore, identification of each spots by MS would shed light on maker proteins for each JS stages.

P2566/B824

Luteinizing Hormone Receptor and Natriuretic Peptide Receptor 2 Guanylyl Cyclase Communication in Mouse Ovarian Follicles.

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Meiotic arrest and resumption in mammalian oocytes is regulated by two opposing signaling proteins derived from the somatic cells of the follicle: the guanylyl cyclase natriuretic peptide receptor-2 (NPR2), which keeps the oocyte arrested in meiosis by maintaining elevated levels of cGMP, and the luteinizing hormone receptor (LHR), which inactivates NPR2, decreasing the levels of cGMP and in turn restarting meiosis in preparation for fertilization. These two membrane proteins have distinct cellular locations within the follicle, raising the question of how they interact to coordinate their opposing activities. We investigated this by localizing both proteins by immunofluorescence confocal microscopy and serial section immunogold electron microscopy in mouse preovulatory follicles. The LHR is absent from cumulus cells and is localized almost entirely to the outer mural granulosa cells. We found that only about 20-40% of outer mural granulosa cells express the LHR protein. In contrast, NPR2 is expressed in all the follicle cells with a higher concentration in cumulus cells. When protein expression levels are normalized to the size of the follicle, we find that most of the total NPR2 protein is found in the mural granulosa cells, but only 10-20% of the total NPR2 is in cells that also express LHR. These findings indicate that in order to account for the cGMP decrease that occurs in response to LH, the cells that express the LHR must also send a signal that inactivates NPR2 in the neighboring cells which do not express the LHR.

P2567/B825

Proteostasis Renewal in the *C. Elegans* Germline: Identifying Changes in Oocyte Cell Biology Triggered by Signals from Sperm.

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Somatic cells age and die, yet the germ lineage is rejuvenated with each generation, ensuring germline immortality. In *C. elegans*, protein aggregates accumulate in unfertilized oocytes of females, which lack sperm. These aggregates are removed, by a process resembling microautophagy, in response to sperm-derived signals when the animals are mated. Our previous findings indicate that oocytes in the hermaphrodite germline respond to sperm-derived signals by rapid acidification of lysosomes and a subsequent shift in mitochondrial physiology, that are required for aggregate clearance (1). We have performed a genome-wide RNAi screen to identify additional components of this quality control switch, and to explore changes in oocyte cell biology triggered by sperm signals. By knocking down individual genes and monitoring an aggregation-prone protein within maturing oocytes, we have identified about 100 genes necessary for preventing oocyte-protein aggregation when sperm are present. Multiple different organelles and cellular processes are regulated by these genes. Our findings indicate that the endoplasmic reticulum (ER) and its trafficking function are required to acidify lysosomes. We hypothesize that these factors are important for the assembly of the lysosomal V-ATPase proton pump and are conducting experiments to visualize the dynamics of ER and V-ATPase subunits following mating. We are also investigating how other candidates identified in the screen contribute to oocyte proteostasis. Taken together, we hope that this study helps to better understand the natural rejuvenation strategies of the germ lineage. The insights could potentially guide approaches for

activating quality control pathways in somatic tissues during aging. (1) Bohnert KA and Kenyon C. 2017. A lysosomal switch triggers proteostasis renewal in the immortal *C. elegans* germ lineage. *Nature* **551**:629-33

P2568/B826

The Inner Nuclear Proteome Is Dynamic.

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Changes in the inner nuclear membrane (INM) proteome have been proposed to occur during development, differentiation, and numerous disease processes. However, how the INM proteome changes and how these changes are coupled with nuclear function remains poorly understood. In addition, the timing of when these changes happen at the INM remain unknown although it is reasonable to speculate that it coincides with its function at the INM. We previously developed a system using bimolecular fluorescence to study the INM proteome in mitotic cells. Here, we adapted the split-GFP system to study the INM during gametogenesis. In yeast cells, following two meiotic cellular divisions, newly formed haploid genomes are each encapsulated by a spore wall within the mother cell. This highly regulated developmental process, sporulation, leads to the production of four gametes from the mother diploid cell. We observed specific changes in the INM composition during spore formation, including loss and gain of INM components compared to the mitotic INM proteome. We are currently using several strategies to determine when some of these changes occur. Having insight regarding the timing of the protein changes at the INM may give insight into the nuclear function of the change or help to explain why the change is important. Interestingly, we have found that all positive proteins are detected in two of the four spores. Our INM split-GFP cell strains were heterozygous for the tagged gene. The simplest explanation for this distribution pattern is that these INM proteins are made *de novo* in the spores that inherit the tagged gene. Unlike the INM during mitosis, where it is partitioned between the mother and daughter; the unique pattern of INM inheritance that we observe involves spore-specific *de novo* INM assembly. The changes that we observe during gametogenesis at the INM may all be spore-specific rather than being dependent on meiosis I or II.

P2569/B827

Increased Vascular Endothelial Growth Factor by Placenta-derived Mesenchymal Stem Cells Promotes Folliculogenesis in Ovariectomized Rat Model.

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Primary ovarian insufficiency (POI), also known as premature ovarian failure (POF), is one of the most prevalent pathologies for women at reproductive age. The symptoms include reduced number of follicles and diminished secretion of the reproductive hormones before reaching the age of 40. An d the conventional treatment for the dysfunction focuses on hormonal therapy, most commonly hormone replacement therapy (HRT). However, due to its limitation of side effect risks such as heart disease and osteoporosis, an alternative treatment method is desirable, and, thus, studies on stem cell therapy have rapidly advanced. Recent studies reported that transplantation of human mesenchymal stem cells (MSCs) from various sources has promoted ovarian regeneration. However, not many studies were conducted in regards to the effect of placenta-derived mesenchymal stem cells (PD-MSCs), and its mechanism of the ovarian recovery remains to be elucidated. PD-MSCs are derived from the chorionic

plate of the human placenta and are known to promote angiogenesis and have therapeutic effect of liver regeneration. Here, we investigated the restorative effect of PD-MSCs on folliculogenesis using ovariectomized (OVX) rat model. $5x10^5$ of PD-MSCs were intravenously injected into OVX rat models and after 5 weeks the animals were sacrificed for analysis of their serum, genes and proteins. The engraftment and localization of PD-MSCs to the ovaries were confirmed by the expression of *hAlu* upto 5 weeks after the transplantation. ELISA analysis of the serum revealed that the group treated with MSCs for 3 weeks showed decreased level of estradiol (E2) compared to the non-treatment group ($P<0.05$). In addition, histological analysis showed the treatment group had increased number of follicles than the non-treatment group ($P<0.05$), along with the increased number of antral follicles ($P<0.05$), contributing 2-fold increase. Furthermore, the elongated and expanded arteries in ovary were restored with reduced thickness of the wall and decreased luminal area as observed in the wild-type group ($P<0.05$). To investigate the mechanism, cytokine assay was performed and discovered elevated secretion of vascular endothelial growth factor (VEGF) by PD-MSCs, and the increased VEGF secretion was confirmed in the serum of the OVX models. Also, high expression of vascular endothelial growth factor receptor 2 (VEGFR2) was detected through western blot and immunofluorescence at the arteries in ovary. In summary, these findings suggest that the engrafted PD-MSCs secreted VEGF and, thereby, enhanced follicular development in OVX model, providing a theoretical foundation for their application.

P2570/B828

Colocalization and Functional an alysis of *Arabidopsis Thaliana* Tts Protein.

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Colocalization and Functional an alysis of *Arabidopsis Thaliana* TTS Protein Arabinogalactan proteins (AGPs) are a superfamily of glycoproteins in plants. They have been implicated with important roles in plant growth, development and reproduction. Several AGPs are expressed in pistils, the female organs of plants, and perform important functions in the fertilization process. Examples of these are the Transmitting Tissue-specific (TTS) proteins from tobacco plants, which stimulate and promote pollen tube growth in plants, and attract them to their target ovules where fertilization takes place. TTS-1, 2 are located in the extracellular matrix that form the transmitting tissue inside the pistils, where pollen tubes grow and transport sperm for fertilization. Recently, we have identified three homologs of the tobacco TTS in *Arabidopsis thaliana* (At), which is considerably more amendable for genetic analysis than tobacco. We refer to these TTS genes as AtTTS-1, 2, 3. The focus of the project is to determine the function of the AtTTS gene family (i.e. TTS-1,-2,-3) in *Arabidopsis Thaliana*. Initial characterization of T-DNA induced knockout mutants of AtTTS-1, 2 did not reveal reproductive defects. Additionally, RNAi construct for these two genes induced reduced fertility. Our hypothesis is that the RNAi may have targeted other TTS-related genes and we have identified a potential candidate, AtTTS-3 by virtue of its high homology with AtTTS-1, -2 and with the original tobacco TTS-1, 2. We have obtained two T-DNA insertion mutant lines in AtTTS-3. Currently, we are characterizing these mutant lines. We will examine the mutant lines reproductive phenotypes to compare with the At-TTS-1, -2 RNAi and generate double or triple mutants with tts-3. In addition, we shall carry out varying assays, these will be conducted to reveal information about fertility properties, such as monitoring seed setting, in *vivo* and semi-*in vivo* pollen germination and tube growth assays.

P2571/B829

Proteins Released from the Egg Cell Surface at Fertilization in Sea Star.

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One of the biggest challenges facing the egg during fertilization is the ability to fuse with a single sperm while blocking all others. There are two clearly documented methods that the egg uses to protect against polyspermy; the fast block, in which the egg cell surface depolarizes to prevent further sperm binding, and the slow block, due to cortical granule exocytosis during egg activation, which raises the fertilization envelope creating a stable and permanent barrier around the egg. Juno and Izumo1, the first fertilization-critical sperm-egg binding partners, were recently discovered. Juno, the egg membrane protein, was discovered to be shed off of the surface after fertilization, perhaps as another mechanism to prevent polyspermy. Shedding of membrane proteins has been documented in other cell types and a variety of processes as well, including the initiation of signaling processes. To this effect, I have observed peptide shedding during egg activation in the sea star *Patiria miniata*. This system offers a unique opportunity to elucidate the molecules that may be required for sperm-egg fusion and activation of the signaling cascade that leads to calcium release at fertilization. Using *P. miniata* as the model organism, egg cell membrane proteins were labeled with biotin, fertilized, and the surrounding seawater was collected. An y biotinylated peptides that are released from the surface were concentrated using biotin-streptavidin affinity interaction. The eluted proteins are subjected to western-blotting probing for biotin. Biotinylated proteins were shown to be released into the seawater around unfertilized eggs, along with a distinct increase in biotinylated proteins in the seawater after fertilization. These proteins range from approximately 50 to 250 kDa, with several unique bands presenting after fertilization. The seawater eluate from unfertilized and fertilized eggs was subjected to tandem mass spectrometry to identify the released proteins. However, identification is a challenge in this particular model organism because there is no proteomic database available. To overcome this limitation, we developed a mature egg transcriptome (NCBI BioProject: 398668), which was used to identify the peptides detected via tandem mass spectrometry by aligning them to an in silico proteome derived from the open reading frames within the transcriptome. A total of 175 transcripts were identified in the seawater samples; 60 were unique to the unfertilized egg, 78 to the fertilized egg, and 38 transcripts were found in both samples. Using these identified transcripts, proteins that are potentially critical for fertilization can be identified, which can lead to a better understanding of what may be happening at the surface of the egg during this vital step to creating life.

P2572/B830

Genomic and Proteomic Identification of the Signaling Complex Controlling Egg Activation in the Sea Star, *Patiria Miniata*.

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Fertilization is the process of sperm and egg fusing to produce a zygote. Despite being studied for more than 30 years, the signaling pathway controlling egg activation has yet to be fully described in any species. One of the most well-established egg activation pathways is that of sea urchins and sea stars, in which sperm-egg interaction leads to activation of two Src family kinases (SFKs) and phospholipase Cγ (PLCγ). PLCγ cleaves membrane-bound PIP_2 into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), and IP_3 binds to its receptor on the endoplasmic reticulum causing an internal calcium release. This internal calcium release stimulates the permanent block to polyspermy and reinitiates the cell cycle. The

PLC protein and the internal calcium release are components of the egg activation pathway shared across species; however, the isoform of PLC varies, for example PLCγ is responsible in sea stars, while PLCζ is responsible in mammals. PLC proteins are pivotal in prompting this internal calcium release in many signaling pathways and are components of larger signaling complexes transducing this signal from an activated membrane receptor. PLCγ can be activated by receptor or non-receptor tyrosine kinases through phosphorylation of Y783 located between the N-terminal SH2 domain and the SH3 domain. In an effort to identify more of the signaling complex responsible for stimulating and transducing this signal, proteins binding to the tandem PLCγSH2 domains during egg activation in the sea star model system have been identified. Furthermore, since tyrosine phosphorylation is pivotal for activation of the SFKs and PLCγ that are involved in egg activation in sea stars, the global tyrosine phosphorylation changes during egg activation and all of the SH2-domain containing transcripts within the mature, unfertilized egg will be examined. From the combination of these studies, we are narrowing down the potential proteins that may be functioning in the signaling pathway to calcium release during egg activation in the sea star, *Patiria miniata*. These fundamental studies will contribute to identifying new targets for contraception development, as well as, new infertility diagnostics and treatments.

P2573/B831

Transcriptional Activity and Morphological Changes of Somatic Cell Nuclei Transferred into Germinal Vesicle Stage Oocytes.

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Mammalian oocytes become transcriptionally silent during the germinal vesicle (GV) stage, and arrest at the metaphase of second meiosis (MII) until fertilization. The nuclear activities and dynamics during this process are unique to female germ cells and never seen in somatic cells. To understand the mechanisms to make the differences between germ cells and somatic cells, we have attempted to induce meiotic events in somatic nuclei. In this study, mouse or guinea pig fibroblast nuclei were transferred into enucleated mouse or porcine GV stage oocytes, and the reconstructed oocytes were matured in vitro. The nuclear activities especially transcriptional activity and spindle formation were analyzed by the incorporation of ethynyl uridine (EU) and immunostaining for tubulin, respectively. For the EU incorporation analysis, the reconstructed oocytes were cultured in vitro with cAMP to prevent GV breakdown and entry into metaphase, so that we were able to observe nuclei until 15 hours after the initiation of in vitro maturation (hours post maturation, hpm). The incorporation of EU into the nuclei of reconstructed oocytes was evaluated in every 3 hour-periods from 0-3 hpm to 12-15 hpm. The reconstructed oocytes received G0 phase cells had the transcriptional activity until 9-12 hpm, and gradually lost it at 12-15 hpm, while those received M phase cells didn't show the transcriptional activity at any time point observed, suggesting that the cytoplasm of GV stage oocyte suppresses the transcriptional activity of the somatic cell nucleus in cell cycle-dependent manner. The ratio of transcriptionally active oocytes to inactive ones in the control GV stage oocytes didn't change from 0-3 hpm to 12-15 hpm, possibly because the transcriptional status of each oocyte didn't change in the presence of cAMP. This suggests that the cytoplasm of GV stage oocyte doesn't affect the transcriptional status of germ cell nucleus in the presence of cAMP. For immunocytochemistry for tubulin, reconstructed oocytes were cultured without cAMP, and the localization of chromosomes and tubulin

was analyzed. Reconstructed oocytes using mouse GV oocytes had chromosomes spread to entire cytoplasm. Though tubulin was detected around chromosomes, none of reconstructed oocytes had the spindles. Reconstructed oocytes using porcine oocytes also failed to form the spindle, but interestingly, chromosomes didn't spread and existed as an aggregate near the cortical region of the oocyte. These results suggest some species-dependent mechanisms to form the meiotic spindle. Taken together, the somatic nucleus behaves differently from the germ cell nucleus in the cytoplasm of the GV stage oocyte in the manner dependent on species of the oocyte.

P2574/B832

Nuclear Reprogramming of Interspecies Somatic Cell Nuclear Transfer in A*Danionin* Genus Model.

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Mechanisms underlining animal oocytes capabilities to be powerful nuclear reprogrammers can be harnessed by Somatic Cell Nuclear Transfer (SCNT) for studies of key molecular events involved in embryonic development to generate pluripotent stem cells and tissues for transplantation. Limitations of SCNT regarding lack of access to enough high-quality oocytes and ethical considerations, alternative methods to study these key molecular events have been established. A derivative method called interspecies somatic cell nuclear transfer (iSCNT) has been developed in which a somatic cell nucleus is transferred into an enucleated donor oocyte obtained from a relative species. The outcomes of this process are nucleo-cytoplasmic hybrids (cybrids) having the nucleus of one species and the cytoplasm of another. Molecular boundaries in this method involve promoting functional interactions between the donor nucleus and the host egg (i.e. nuclear reprogramming). Phylogenetic parameters dictate these boundaries. Understanding these parameters are imperative to drive forth the use of iSCNT in translation applications. The model developmental system, zebrafish (*Danio rerio*), along with an array of additional members of the *Danionin* genus, including *D. kyathit , D. Albolineatus*, *D. margaritatus* are to be used as a "model genus" for testing nucleo-cytoplasmic incompatibilities of iSCNT cybrids at the key developmental timepoint, the mid-blastula transition (MBT). *We hypothesize that changes in key cellular players driving MBT, acquired during the divergence of two species, will result in defects in gene network activation and cybrid incompatibility*. Cybrids are generated via UV irradiation of zebrafish eggs subject to in *vitro* fertilization using sperm of related species. The embryos are assessed via live imaging to record developmental staging and to track cell cycling. The cybrid embryos that have been generated are: *D. kyathit, D. Albolineatus, and D. margaritatus*. Normal developmental staging of zebrafish is recapitulated by all cybrid embryos in early developmental timepoints prior to MBT. Following MBT (4 hours post-fertilization (hpf)), the *D.* kyathit cybrids continued to exhibit no changes in cell cycling. *D. Albolineatus and D. margaritatus* cybrid embryos exhibited elongated cell cycles and delay in developmental staging. Developmental defect was observed in 100% of *D. margaritatus* cybrids by 12 hpf. The other cybrid groups survived up to 72 hpf. Our data suggest that nucleo-cytoplasmic incompatibility of cybrids is directed by phylogenetic relatedness. The more proximally related *D. kyathit* cybrids demonstrated indistinctive development to zebrafish, while the distally related species, *D. Albolineatus* and *D. margaritatus*, exhibited developmental defect.

Viruses

P2575/B834

Involvement of a Membrane Curving Protein in HIV-1 Gag Assembly.

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The I-BAR proteins are a family of proteins known to sense and induce negative curvature at the cell plasma membrane. They also act as scaffold proteins for several actin signalling pathways at the cell membrane. Our lab have revealed that by siRNA knockdown that I-BAR1, an I-BAR domain protein, involved in HIV-1 Gag assembly via a small GTPase dependent signalling. Hence we examined the role of I-BAR1 in HIV-1 Gag assembly. During HIV-1 assembly and budding, the structural Gag proteins are targeted to the plasma membrane where they oligomerize on the viral genome and the cell membrane, leading to the virus budding from the cell membrane. Here, we confirmed the effect of siRNA mediated gene inhibition of I-BAR proteins on Gag VLP production in 293T cells and wild-type HIV-1 production in host CD4 T cells. Secondly, we analysed the possible interactions between Gag and I-BAR proteins by immunoprecipitation. Then we examined the incorporation of the I-BAR1 protein in infectious wild-type HIV-1 as well as Gag VLPs produced in 293T cells. We tested the specificity of this incorporation across other I-BAR family proteins. Our results show that inhibition of I-BAR1 by siRNA leads to a significant reduction in VLP production. An interaction between Gag and I-BAR1 was established in cells by IP. We then performed studies of purified HIV-1 Gag and I-BAR1 domain in a cell free system to analyse their molecular interplay. Our results indicate an enhancement of Gag binding to lipid membranes in the presence of I-BAR1 domain indicating that preferential binding of Gag to membranes with I-BAR1 clusters, and more so when the membrane is curved. In addition, imaging and gradients of purified VLPs showed that I-BAR1 is specifically incorporated into Gag VLPs as compared to other I-BAR family proteins, and is likewise incorporated in infectious purified HIV-1 particles. We further compared the localization of 2 I-BAR proteins at hundreds of virus budding sites by dual colour super resolution PALM/dSTORM microscopy. Imaging analyses indicated the presence of IBAR-1 in close proximity to Gag assembly sites as compared to I-BAR2. Taken together, our results point towards a novel role for I-BAR1 in HIV-1 Gag particle assembly where it may aid Gag binding to the cell membrane providing the initial cluster and curvature necessary for bud formation.

P2576/B835

Single-cell Glycolytic Activity Regulates Membrane Tension and Hiv-1 Fusion.

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There has been resurgence in determining the role of host metabolism in viral infection yet deciphering how the metabolic state of single cells affects viral entry and fusion remains unknown. Here, we have developed a novel assay multiplexing genetically encoded biosensors with single virus tracking (SVT) to evaluate the influence of global metabolic processes on the success rate of virus entry in single cells. We found that cells with a lower ATP:ADP ratio prior to virus addition were less permissive to virus fusion and infection. These results indicated a relationship between host metabolic state and the likelihood for

virus-cell fusion to occur. SVT revealed that HIV-1 viruses were arrested at hemifusion in glycolytically-inactive cells. Interestingly, cells acutely treated with glycolysis inhibitor 2-deoxyglucose (2-DG) become resistant to virus infection and also display less surface membrane cholesterol. Addition of cholesterol in these in glycolytically-inactive cells rescued the virus entry block at hemifusion and enabled completion of HIV-1 fusion. Further investigation with FRET-based membrane tension and membrane-order reporters revealed a link between host cell glycolytic activity and host membrane order and tension. Indeed, cells treated with 2-DG possessed lower plasma membrane lipid order and higher tension values, respectively. Our novel imaging approach that combines lifetime imaging (FLIM) and SVT revealed not only changes in plasma membrane tension at the point of viral fusion, but also that HIV is less likely to enter cells at areas of higher membrane tension. We therefore have identified a connection between host cell glycolytic activity and membrane tension that influences HIV-1 fusion in real-time at the single-virus fusion level in live cells.

P2577/B836

Visualization of HIV-1 Virus-like Particles at Nuclear Pore Complexes by Cryo-Electron Tomography in *Cellulo*.

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Human Immunodeficiency virus type 1 (HIV-1) is a lentivirus that infects non-dividing cells of the immune system and causes acquired immune deficiency syndrome (AIDS). Replication of the virus is dependent on nuclear entry of the HIV-1 pre-integration complex (PIC) consisting of the reverse-transcribed genome, viral capsid proteins (CA) and integrase (IN), and the subsequent integration of the viral DNA into the host genome. In non-dividing-cells nuclear import occurs through the nuclear pore complex (NPC), a large macromolecular assembly that forms a channel across the nuclear envelope, and requires interactions between viral and host factors. It is generally accepted that HIV-1 CA play a key role in HIV-1 nuclear entry by interacting with NPC components, notably Nup358 and Nup153, and nuclear and transport factor such as CPSF6 and TNPO3. Single mutations in the HIV-1 CA, such as N74D and A77V, abrogate these interactions and interfere with multiple steps of HIV-1 infection. In the mature HIV-1 ~1500 CA assemble into ~200 hexamers and 12 pentamers to form a cone-shaped capsid core. It is not known yet, however, whether the CA of the PIC, reaching the nuclear periphery and interacting with the NPC, keep the same degree of assembly as in the mature capsid core, or whether it is partially or completely lost in a process known as “uncoating”. In order to study the mechanism of nuclear entry and timing of HIV-1 CA uncoating from a biological and structural point of view, we applied cryo-focused ion beam (FIB) milling of T-helper cells transduced with HIV-1 virus-like particles (VLPs) carrying a A77V mutation in the CA followed by cryo-electron tomography (Cryo-ET) to visualize HIV-1 particles interacting with the NPC in *cellulo*. 3D Cryo-correlative light electron microscopy (Cryo-CLEM) was used to validate the nature of the identified particles. Furthermore, we performed subtomogram averaging to define the ultrastructure of HIV capsid-derived structure at the NPC. Our preliminary data show capsid-like structures at the cytoplasmic and the nuclear side of NPCs that resemble the capsid core in shape and size. These findings open new avenues to studying the NPC structural modification during PIC translocation.

P2578/B837

Using Polarization to Image Structural Changes in HIV-1 Maturation.

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The HIV-1 virus life cycle requires a maturation step where a newly made virus is cleaved by the genetically encoded HIV-1 protease. This maturation step is necessary for infectivity, but the exact kinetics of maturation during assembly of new viruses is unclear. We are using polarized light microscopy to determine the kinetics of the HIV-1 protease in live cells that are assembling new HIV-1 viruses. Polarized light microscopy makes use of the following facts: 1) fluorophores have an excitation dipole, and the fluorophore is excited by light parallel, but not perpendicular to the dipole; 2) fluorophores emit light polarized parallel to their emission dipole; 3) generally the emission dipole is roughly co-aligned with the excitation dipole. We are using anisotropy which is a form of polarization imaging that detects changes in the arrangement of the protein complex that a fluorophore is attached to and how dynamic the fluorophore is. Here we show fluorescence polarization can be used to assay structural differences due to active protease-driven maturation of HIV-1 virus-like particles both in collected supernatant and during active assemblies.

P2579/B838

Zika Virus Enhances Monocyte Adhesion and Transmigration, Favoring Viral Dissemination to Neural Cells.

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Zika virus (ZIKV) invades and persists in the central nervous system (CNS), causing severe neurological diseases. However, the virus journey, from the bloodstream to tissues through a mature endothelium, remains unclear. Here, we show that ZIKV-infected monocytes represent suitable carriers for viral dissemination to the CNS using human primary monocytes, cerebral organoids derived from embryonic stem cells, organotypic mouse cerebellar slices, a xenotypic human-zebrafish model, and human fetus

brain samples. We find that ZIKV-exposed monocytes exhibit higher expression of adhesion molecules, and higher abilities to attach onto the vessel wall and transmigrate across endothelia. This phenotype is associated to enhanced monocyte-mediated ZIKV dissemination to neural cells. Together, our data show that ZIKV manipulates the monocyte adhesive properties and enhances monocyte transmigration and viral dissemination to neural cells. Monocytes transmigration may represent an important mechanism requires for viral tissue invasion and persistence that could be specifically targeted for therapeutic intervention.

P2580/B839

Ifitm3 Inhibits Retroviral Infectivity by Rerouting Viral Envelope Glycoproteins to Lysosomes.

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Interferon-induced transmembrane (IFITM) proteins are encoded by many vertebrate species and exhibit antiviral activities against a wide range of viruses. IFITM proteins, particularly IFITM3, perform multiple antiviral functions during HIV-1 infection. First, IFITM3 prevents virus-cell fusion when expressed in naïve target cells. Second, when present in virus-producing cells, it reduces the fusion potential of virus particles. This latter activity was first characterized during HIV-1 infection, and the mechanism responsible may involve IFITM protein incorporation into virions, reduction of Envelope (Env) incorporation, or both. To address the breadth of antiviral activity exhibited by IFITM3 as well as the underlying mechanism(s) involved, we took advantage of a murine leukemia virus (MLV)-based pseudotyping system. By carefully controlling quantities of IFITM3 and viral glycoproteins in virus-producing cells, we found that ectopic IFITM3 potently inhibits MLV virion infectivity, and most potently when Env levels are limiting. Loss of virion infectivity was associated with defective proteolytic processing of Env, lysosomal degradation of the Env precursor, and a paucity of Env incorporated into virions. Ecotropic and Xenotropic variants of MLV Env, as well as HIV-1 Env and VSV-G, are sensitive to IFITM3 while Ebola glycoprotein is resistant, suggesting that IFITM3 selectively targets certain viral glycoproteins. Furthermore, endogenous IFITM3 in human and murine cells negatively regulates MLV Env production. These data indicate that IFITM3 decreases virion infectivity in a viral glycoprotein-dependent manner, and it does so in part by limiting viral glycoprotein production. However, by titrating Env levels during virus production, we found that the negative impact of IFITM3 on virion infectivity is greater than the impact of decreasing Env incorporation, suggesting that IFITM3 also affects Env qualitatively. Lastly, we demonstrate that virion infectivity in the presence of IFITM3 is rescued by the expression of GlycoGag, a murine retrovirus accessory protein previously described to antagonize the antiviral activity of other intrinsic immune factors SERINC3 and SERINC5. Overall, we show that IFITM3 impairs virion infectivity by regulating Env abundance and function but that enhanced Env expression and GlycoGag confer resistance to IFITM3.

P2581/B840

Influenza Versus Host: Interferon an tagonism by Viral Nucleoprotein and Novel Copper-related Host Factors in Antiviral Defenses.

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Influenza a virus is a respiratory orthomyxovirus that causes 3-5 million cases of severe illness and 250,000-500,000 deaths annually in humans. The NS1 protein antagonizes RIG-I and IRF3-induced IFN-βresponses during an influenza infection. Some influenza strains also contain nucleoproteins (NP)

variants that impart resistance to the antiviral protein Mx. To better understand roles of NP in antagonizing interferon responses, NP plasmids from H1N1, H5N1, or H7N9 influenza subtypes were transfected into A549 human lung adenocarcinoma and HEK293T IFN-βreporter cells. The viral RNA mimic poly-I:C was used to induce a type-I IFN response. Quantitative RT-PCR showed that NP expression led to a 16-fold decrease in IFN-β expression compared to control, and significant reductions in interferon-stimulated genes MxA (185-fold), ISG56 (450-fold), and IL-8 (32-fold). On the host side, copper handling machinery has been recently recognized as regulating influenza infection. RNA interference experiments showed that copper transporter ATP7A, a protein that imports [Cu1+] into trans-Golgi derived compartments, is required for influenza viral RNA and protein syntheses. The trafficking-regulatory Commander complex includes the 10-member COMMD protein family, first identified in regulation of copper export. Preliminary experiments reveal that a type I IFN response induced by poly-I:C in A549 cells also results in dose-dependent upregulation of COMMD5 (13-fold) and COMMD7 (12-fold), but not COMMD1. While simultaneous knockdown of COMMD5 and COMMD7 did not enhance influenza A/WSN/33 (H1N1) infection of A549 cells, knockdown of the copper-binding protein ATOX1 licensed viral replication in a limiting dilution assay. Taken together, these results suggest a previously unrecognized interplay between viral NP, antiviral proteins and copper-binding proteins to regulate influenza a virus infection in mammals, providing novel targets for antiviral therapy.

P2582/B841

The Functional Relationship between Antiviral Restriction Factor IFITM3 and Cellular Cholesterol Homeostasis.

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The interferon-induced transmembrane proteins (IFITM) proteins broadly inhibit infection by multiple viruses, including Influenza a virus, Zika virus, and HIV-1. Previous reports illustrated that IFITM3 perturbs cholesterol trafficking in cells but how it contributes to its antiviral functions is unclear. We confirmed that cholesterol levels are elevated in cells ectopically expressing IFITM3, particularly in the endocytic compartment. Furthermore, we show that increases in cellular cholesterol are associated with increases in membrane order and rigidity using fluorescence lifetime imaging microscopy. Surprisingly, a single point mutation in the intracellular loop of IFITM3 prevents the observed increases in cholesterol and membrane order while concomitantly reducing antiviral activity against Influenza a virus and HIV-1. We found that nystatin and amphotericin B, two antifungal compounds which displace membrane cholesterol, counteracted both the antiviral function of IFITM3 and its capacity to increase membrane order, further linking the antiviral phenotype with membrane lipid composition. To further understand how IFITM3 expression leads to increased intracellular cholesterol concentrations, we used immunoprecipitation and mass spectrometry to identify binding partners that differentially bind wild-type and mutant IFITM3. Initial analyses revealed that wild-type IFITM3 interacts with the rate-limiting enzyme of cholesterol biosynthesis known as SQLE, whereas mutant IFITM3 interacts to a lesser extent. Furthermore, bioinformatic analysis identified a potential cholesterol binding motif which overlaps with the single point mutation present in mutant IFITM3 and we are performing in *vitro* experiments measuring its direct cholesterol binding capacity. We will present experiments using RNAi and specific inhibitors to determine whether SQLE expression is required for IFITM3-mediated antiviral activity. Our

studies will determine the mechanism by which IFITM3 inhibits virus entry and will identify an important metabolic axis that may be targeted for host-directed antiviral therapy.

P2583/B842

The Multiple Roles of Canine Parvovirus Non-structural Protein 2.

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Canine parvovirus (CPV) is a small DNA virus with only four virally encoded proteins. Viral proteins 1 and 2 form the capsid core, while non-structural protein 1 performs a number of nuclear functions required for viral replication. The exact role of the fourth CPV protein, non-structural protein 2 (NS2), has remained undefined. Here, we used BioID combined with confocal microscopy and other protein-protein interaction analyses to identify binding partners for NS2. Our results revealed multiple NS2 - host protein associations, which could have a significant impact on the interplay between virus and cell machinery and thereby influence CPV replication. NS2 was found to be associated with the key factors that control chromatin remodeling, presumably either to promote viral replication or to inhibit cellular functions that counteract infection. An alyses of the factors involved in DNA damage response and transcription showed that NS2 is associated with regulators of cell cycle arrest and apoptosis. Finally, NS2 appears to be involved in splicing, binding and NPC docking of mRNA, thereby contributing as an important mediator and effector in mRNA processing and nuclear export. Our analyses reveal novel data of the transient interactions and implicated mechanisms by which parvoviral NS2 could operate in infection.

P2584/B843

CRISPR/Cas9-Mediated Knockout of the Coxsackievirus and Adenovirus Receptor in Polarized Epithelial Cells.

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Adenovirus (AdV) infection generally causes cold-like symptoms, but in some cases can be severe and can be fatal. The Coxsackievirus and adenovirus receptor (CAR) is encoded by the *CXADR*gene and the transcript is alternatively spliced yielding two transmembrane protein isoforms of CAR, CAR^{Ex7} and CAR^{Ex8}. The only difference between the two isoforms is at the extreme C-terminus where the last 26 amino acids of $CAR^{Ex7}$is replaced by 13 unique amino acids encoded by the eighth exon of *CXADR*. This sequence difference results in distinctly different localization for each isoform in polarized epithelial cells. Whereas CAR^{Ex7} is found on the basolateral surface, CAR^{Ex8} is localized at the apical surface where it can mediate the infection of AdV from the airway lumen. We hypothesized that isoform-specific knockout of CAR^{Ex8}would prohibit apical AdV infection while the knockout of both isoforms would prevent apical and basolateral AdV infection. To test this, we used CRISPR/Cas9 to target CAR^{Ex8}or all CAR isoforms and then investigated AdV5-LacZ entry and transduction. We previously had established a Madin-Darby canine kidney cell line that has a stably-integrated lentiviral-based dox-inducible human CAR^{Ex8}insert (MDCK-CAR^{Ex8}). We used this cell line to create two CRISPR/Cas9 edited MDCK-CAR^{Ex8} cell lines, one with CAR^{Ex8}knockdown (JR1) and one total CAR knockout (AA1). Knockdown/knockout of

CAR^{Ex8} andtotal CAR significantly decreased apical AdV transduction in comparison to parental cells demonstrating the importance of the apical isoform for AdV infection. This effect was reversed and infection was restored with dox-mediated induction of the lentiviral CAR^{Ex8}insert. Future work will evaluate basolateral infection and investigate whether AdV can exploit additional mechanisms to enter into polarized epithelia.

P2585/B844

Contributions of the Four Essential Entry Glycoproteins to HSV-1 Tropism and the Selection of Entry Routes.

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Herpes simplex virus type 1 (HSV-1), an enveloped virus that is a member of the herpesvirus family, can enter cells by either fusion at the plasma membrane or endocytosis, depending on the cell type being infected. However, it remains unclear how this choice is made. In order to enter a cell, HSV-1 requires four of its 15 total envelope proteins: gB, gH, gL, and gD. While these are the only four proteins required for entry and membrane fusion, their contributions to the ability of HSV-1 to infect different cell types and the selection of different entry routes remains unclear. To address the specific contributions of gB, gH, gL, and gD to HSV-1 cellular tropism and different entry pathways, we generated vesicular stomatitis virus (VSV) lacking its native glycoprotein G, and pseudotyped it with gB, gH, gL, and gD, which we termed VSVΔG-BHLD virions. Six HSV-1 susceptible cell lines were then screened for their ability to support VSVΔG-BHLD virion entry. Out of those six cell lines, only two supported VSVΔG-BHLD entry: C10 and CHO-nectin-1 cells. In those two cell lines, HSV-1 and VSVΔG-BHLD entry mechanisms were determined using a combination of redundant chemical and genetic inhibitors of cellular uptake pathways. Our results show that VSVΔG-BHLD virions lack the broad cellular tropism that HSV-1 possesses. Additionally, VSVΔG-BHLD virions utilize different entry pathways compared to HSV-1 in C10 and CHO-nectin-1 cells. While HSV-1 utilized an entry pathway in both cell lines that was dependent on clathrin, dynamin, cholesterol, and Arf6, and independent of endosomal pH and Rab5 or Rab7 function, VSVΔG-BHLD virions entered C10 and CHO-nectin-1 cells by a clathrin- and Arf6-independent mechanism that was dependent on dynamin and cholesterol. Endosomal pH and Rab5 and Rab7 GTPases were important for VSVΔG-BHLD virion entry in a cell-line dependent manner. Thus, while the four essential HSV-1 entry glycoproteins enable entry in certain contexts, they are insufficient for entry into any HSV-1-susceptible cell and do not specify native entry routes. We conclude that the HSV-1 envelope proteins outside the essential four (so-called “non-essential”) contribute towards the tropism and the selection of entry routes. Our work draws attention to the need for systematic investigation of the HSV-1 entry mechanisms and the roles of the envelope proteins long considered non-essential in the selection of target cells, routes of entry, and pathogenesis.

P2586/B845

HIV-1 Envelope Glycoprotein Trafficking and Viral Transmission.

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HIV-1 encodes an envelope glycoprotein complex (Env) containing a long cytoplasmic tail (CT) harboring trafficking motifs implicated in Env incorporation into virions. Although the requirement for the Env CT in viral transmission is known, the precise mechanism by which Env is incorporated into nascent virions and localizes to the virological synapse remains poorly defined. To further elucidate the mechanism of Env trafficking we examined two HIV-1 strains: the lab-adapted clade B strain, NL4-3, and a transmitted/founder clade C virus, K3016. The HIV-1 Env CT contains a highly conserved tyrosine endocytosis motif, Y^{712}SPL, and C-terminal dileucine motif, LL^{855}. Virion Env incorporation analysis revealed that Y^{712}SPL is necessary for efficient Env incorporation while LL^{855} is dispensable. Spreading infection kinetics were analyzed in various T-cell lines and primary human PBMCs; the results indicated that both endocytic motifs contribute to efficient viral spread in culture. An alysis of Env localization to the T-cell uropod, the portion of the plasma membrane that forms a virological synapse with uninfected cells, was found to be dependent on the Env CT and the Y^{712}SPL motif. Cell-to-cell and cell-free transmission assays using T cells infected with HIV-1 bearing Y_{712}A or LL_{855}AA Env CT mutations are ongoing to establish a role for these motifs in both modes of viral transmission. These studies will significantly enhance our understanding of Env trafficking and viral transmission, providing insights into viral Env-host interactions in physiologically relevant cells.

P2587/B846

DLK/JNK-dependent Reactivation of Herpes Simplex Virus from Latency in Response to Neuronal Hyperexcitability.

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Herpes simplex virus (HSV) establishes a latent infection in peripheral neurons. The viral DNA episome persists in the nucleus and assembles into heterochromatin resulting in silencing of HSV lytic genes. In response to certain stimuli, viral lytic gene expression initiates, which can result in production of viral particles and reactivation from latency. The exact physiological triggers contributing to reactivation are not well understood. Previously, we found that dual leucine zipper kinase (DLK)-mediated activation of c-Jun N-terminal kinase (JNK) was essential for reactivation in response to inhibition of PI3K signaling, which mimics loss of nerve growth factor (NGF) support. Moreover, JNK activation in sympathetic neurons was associated with a histone phospho/methyl switch on HSV lytic gene promoters, therefore linking NGF signaling to epigenetic changes on the viral genome that would permit gene expression. Given that the same histone phospho/methyl switch occurs in cortical neurons following hyperexcitability (triggered by forskolin or KCl depolarization) we examined whether HSV reactivation was linked to hyperexcitability and the contribution of JNK activity and histone phosphorylation. Using a model of HSV reactivation in mouse sympathetic neurons, we found that forskolin induces HSV lytic gene expression and reactivation in a DLK/JNK-dependent manner. The initial burst of lytic gene expression occurred independently of histone demethylase activity and was accompanied with a transient histone phospho/methyl switch. Reactivation in this context was not linked to loss of AKT phosphorylation. In addition, forskolin and inhibition of PI3K signaling synergized to enhance reactivation, indicating that loss of NGF support and forskolin trigger reactivation via distinct pathways that converge on DLK/JNK. Forskolin-mediated reactivation of HSV was blocked following treatment with ion channel inhibitors. In addition, hyperexcitability resulting from removal of a tetrodotoxin block triggered HSV reactivation in a DLK/JNK-dependent manner. We next investigated whether physiological

triggers induce HSV reactivation via hyperexcitability. Notably, IL-1β induced DNA damage associated with hyperexcitability in adult neurons. IL-1β also triggers DLK/JNK-dependent reactivation of HSV that was dependent on ion channel activity. Therefore, we posit a model that neuronal hyperexcitability in response to physiological stimuli such as inflammation trigger HSV reactivation. These data also place activation of DLK/JNK and a histone phospho/methyl switch as a key event in hyperexcitability-mediated reactivation.

P2588/B847

The Cell-specific Role of Viral Kinase in the Formation of Tunneling Nanotubes and in Cell-to-cell Spread of an Alpha-herpesvirus.

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The Us3 gene coding for a serine-threonine protein kinase is highly conserved among alphaherpesviruses. The Us3 kinase is a multifunctional protein which plays role in many processes including virus egress from the nucleus, blocking apoptosis and modulation of immune response. Us3 interaction with host cell actin cytoskeleton can lead to dramatic changes in the cell morphology. For our model herpesvirus, bovine herpesvirus 1 (BoHV-1) we reported that overexpression of Us3 in epithelial cells or fibroblasts caused actin stress fibers breakdown and very fast formation of numerous cell projections. This phenomenon was also observed for other alpha-herpesviruses, e.g. pseudorabies virus (PRV). We showed that such projections were produced during natural infection with BoHV-1 and many of these cell connections had features characteristic for tunneling nanotubes (TNT): they contained actin and microtubules, they were long (over 100 nm), thin and fragile. Using fluorescent viral mutants, we demonstrated that viral proteins were transported through TNTs. Moreover, TNTs were used for viral dissemination by cell-to-cell spread—quick, direct intercellular transmission of viral particles. TNTs were detected in uninfected cells permissive for BoHV-1 but virus infection stimulated more than two-fold increase both in number and length of TNTs. That increase was more conspicuous in infected fibroblasts than in epithelial cells. We also observed TNTs formation in human transformed cells which were permissive for BoHV-1. Cells infected with viral mutants lacking Us3 gene or expressing inactive form of this protein generated significantly less connections than cells infected with wild type virus. These data indicate that Us3 kinase plays role in TNTs formation during BoHV-1 infection and contributes to viral spread by cell-to-cell route. In contrast to these data, we discovered that the expression of Us3 in live neurons led to reducing the number of dendrites and, gradually, to cell death. The inactive form of Us3 kinase did not cause such effect. These observation show that viral kinase may interact with host proteins in different ways in various types of cells permissive for herpesviruses. In view of ubiquitous presence of herpesviruses in human and animal populations and the potential use of herpesviral vectors for therapeutic purposes, the precise understanding of molecular mechanisms of herpesviruses interaction with their hosts is of great importance.

P2589/B848

Cpsf6 Accumulation in Speckles After Hiv-1 Infection.

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CPSF6 Accumulation in Speckles after HIV-1 Infection KyeongEun Lee[1], Hyun Jae Yu[1], Stephen H. Hughes[2], and Vineet N. KewalRamani[1] [1]Basic Research Laboratory, National Cancer Institute, Frederick,

MD, [2]HIV Dynamics and Replication Program, National Cancer Institute, Frederick, MD HIV-1 infection at the cellular level involves a successive series of host factor interactions to drive the viral replication program. HIV-1 capsid (CA) plays a central role in these early replication steps facilitating cytoplasmic-nuclear trafficking of the virus and access to chromatin during integration. CPSF6 is as an HIV-1 CA interacting factor that enables HIV-1 integration in gene-rich regions of chromatin. The majority of CPSF6 resides in nucleoplasm with a fraction that also localizes within nuclear speckles. Soon after HIV-1 infection, CPSF6 and HIV-1 begin to accumulate in speckles, such that the majority of CPSF6 is observed within the phase-separated compartments in as little as 6 hours after virus exposure. The enrichment in speckles requires CA interacting with an FG-motif in CPSF6. We find that this localization is essential for HIV-1 targeting of gene-rich regions of chromatin and observe a proximity of speckles to these regions. Notably, while HIV-1 requires CPSF6 to access speckles, when the HIV-1/CPSF6 binding is disrupted with a drug that targets the CA interface essential for interaction, CPSF6 is rapidly flushed from the speckles into the nucleoplasm. Thus, while HIV-1 requires CPSF6 to access speckles, CPSF6 appears to require HIV-1 to persist in speckles. We are currently examining CPSF6 domains to determine whether these phenotypes are genetically and functionally separable. CPSF6 trafficking to speckles in response to HIV-1 infection may provide a model to better understand both the localization and persistence of factors in phase-separated compartments.

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Host-Pathogen / Host-Commensal Interactions 2

P2590/B849

RSV-induced TGFb Plays a Protective Role in Epithelial Barrier Function.

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Respiratory Syncytial Virus (RSV) is a global health and economic burden due to lack of a vaccine or cost-effective antiviral therapy and RSV-associated hospitalization. Studying RSV-host interactions in the human airway epithelium model will likely enhance our understanding of RSV pathogenesis and assist in identifying novel strategies for limiting the severity of RSV-induced pathophysiology. We developed pseudo-stratified and fully differentiated mucociliary epithelium of human bronchial epithelial (NHBE) cells in air-liquid interface 3D culture system. RSV infection in human airway epithelium neither reduced epithelial barrier function nor impaired ciliary function. We found RSV infection increased tumor growth factor-beta (TGFb) induction in NHBE monolayers and pseudostratified airway epithelium. TGFb is known to induce epithelial-mesenchymal transition (EMT). To our surprise, RSV infection in respiratory epithelium neither reduced tight junction markers (E-cadherin and Zonula occludens-1) nor increased mesenchymal cell markers (Vimentin and Alpha-Smooth Muscle Actin). We found that TGFb alone can increase not only ciliary beat frequency but also transepithelial electrical resistance in the airway model. Thus, RSV-induced TGFb may play a protective role in epithelial barrier function in this human airway epithelium.

P2591/B850

Structure-function an alyses of the Small, Non-structural Proteins A30.5 and L2 Provide New Insights into Our Understanding of ER Dynamics and the Formation of the Vaccinia Virus Membrane.

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The choreography of viral infections often sheds light on the cell biology of the host. Vaccinia virus, a complex dsDNA virus, is unusual in replicating exclusively within the cytoplasm of infected cells. Viral replication depends upon dynamic interactions with endoplasmic reticulum (ER) membranes. Viral membrane biogenesis involves diversion of ER membranes, independent of classical vesicular trafficking pathways, leading to the appearance of single-bilayer crescents. These then enlarge to form the delimiting membrane of nascent virions. A30.5 and L2 are small (42 and 87 aa, respectively), non-structural, essential viral proteins that localize to the ER. To better understand how these proteins function, we generated CV1 cell lines that express 3xFLAG-A30.5 or HA-L2 in a DOX-inducible manner, as well as deletion viruses. Loss of either A30.5 or L2 leads to a reduction in viral yield of 2-3 logs and an early arrest in viral membrane biogenesis. We have conducted structure-function studies to identify critical regions within these proteins. A series of truncation, clustered charge-to-alanine, and chimeric alleles were generated and tested in our transient complementation assay. Interestingly, these two proteins are remarkably resistant to perturbation; however, an A30.5 mutant that is predicted to break an N-terminal α-helix and an L2 mutant (RRD^{38,42,46}-AAA), failed to restore viral yield. We have shown that A30.5 and L2 interact with each other in the absence of other viral proteins. Interestingly, the stability of A30.5 is dependent upon the presence of L2, indicating that the vΔL2 mutant actually reflects a loss of both L2 and A30.5. Biological competency of our mutant L2 and A30.5 alleles correlates with their ability to interact. Interestingly, the L2-RRD protein has a significantly retarded mobility compared to L2-WT, which we suspect reflects an aberrant post-translational modification that perturbs L2's biological competency. We are using our cell lines and mutant alleles to monitor ER dynamics through both electron microscopy and immunofluorescence analyses. We have generated cell lines expressing APEX2-A30.5 and -L2 to identify interacting partners of L2 and A30.5 in the absence or presence of infection. In sum, these studies are furthering our understanding of poxviral membrane biogenesis and providing new insight into the dynamic regulation of ER.

P2592/B851

Suppressor of Cytokine Signaling 3 Is Involved in Regulation of Interleukin 6 Expression during the Viral Infection.

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Upon the recognition of viral pathogen associated molecular patterns by pattern recognition receptors, such as toll-like receptors or retinoic acid-inducible gene I-like receptors, various inflammatory cytokines including interleukin 6 (IL-6) are produced in host in response to the infection. However, IL-6-involved excessive inflammatory response to viral infection such as Influenza a virus (IAV) infection profoundly contributes to the virus pathogenesis. The precise mechanisms underlying such a response are poorly understood. Here we found from both in *vivo* and in *vitro* studies that acute infection with IAV not only induced surge of IL-6 release, but also greatly increased expression of suppressor of cytokine signaling 3 (SOCS3), the potent suppressor of IL-6/signal transducer and activator of transcription 3 (STAT3) signaling. Interestingly, there existed a cytokine-independent mechanism of the robust induction of SOCS3 by IAV at early stage of the infection. Furthermore, we employed SOCS3-knockdown transgenic

mice (TG), and surprisingly observed from virus challenge experiments using these mice that disruption of SOCS3 expression provided significant protection against IAV infection, as evidenced by attenuated acute lung injury, a higher survival rate of infected animals and lower viral load in infected tissues as compared with those of wild-type littermates under same condition. The activity of NFκB and the expression of its target gene IL-6 were markedly suppressed in SOCS3-knockdown A549 cells and the TG mice after infection with IAV. Moreover, we defined that enhanced STAT3 activity caused by SOCS3 silencing was important for the regulation of NFκB and IL-6. These findings establish a critical role for SOCS3 in the pathogenesis of IAV through regulating IL-6-STAT3 signaling, and suggest that influenza virus may have evolved a strategy to circumvent IL-6/STAT3-mediated immune response by upregulation of SOCS3.

P2593/B852

Pathogenicity Factors Related to Augmented Virulence of Esbl *Klebsiella Pneumoniae*.

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Abstract the emergence of multi-drug resistance (MDR) for pathogenic bacteria is one of the most serious global threats to human health in the 21st century. In fact, the world health organization (WHO) and the US CDC described the situation as a global crisis and an impending catastrophe of a return to the pre-antibiotic era. The MDR is especially concentrated in bacteria that cause infections associated with health care (HAIs). The global rate of HAIs in the United States is approximately 5% resulting in 88,000 deaths. *K. pneumoniae* is one of the major producers of HAIs and one of the most important bacteria that increasingly acquired antimicrobial drug resistance. Its high resistance does not fully explain its great success as one of the agents HAIs producers, which suggests that other factors facilitate its high frequency. Factors that increase their survival are virulence factors, such as fimbria, siderophores or capsule. In order to find a relationship between virulence factors and the MDR, in this work we analyses 122 randomly selected clinical isolates of *K. pneumoniae*. By PCR, we identify the frequency of the presence of the genes that encode the virulence factors K2, *mag*A, *mrp*A, *ent*B, *ybt*S and *all*S. We also establish their susceptibility profile for the antibiotics; Amk, Cfx, Cfz, Imp, Mer any Pip-taz, by quantification of the minimal inhibitory concentration (MIC). Our results showed a significantly increased frequency of virulence genes in ESBL producing bacteria compared to non-ESBL producing bacteria (Fisher´s; $p<0.026$). We found three of the genes K2, *ybt*S and *all*S that improve the β-lactamase production in the *K. pneumoniae* population. A risk evaluation calculating odds ratio (OR), determinate that the presence of the K2 gene increase 5.9 times (CI: 1.6 – 21), and the gene *ybt*S 3 times (CI 1.2 – 8) the risk to generate ESBL producing bacteria. None of the genes analyses in its study modifies the risk to generate carbapenem resistant bacteria. We observed that all strains of *K. pneumoniae* in or population of the $K2^+$, *ybt*S^+ and *all*S^+ genotype, were ESBL producers. These strains also deploy higher virulence as they shown an increased resistance to human serum, and for human macrophages and polymorphonuclear cells. In sum, the genes $K2^+$, *ybt*S^+ and *all*S^+ provide higher virulence and affect the susceptibility of *K. pneumoniae* improving its resistance to β-lactamic antibiotics.

P2594/B853

Role of Host Exocytosis in Internalin A-mediated Entry of *Listeria Monocytogenes*.

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Listeria monocytogenes (*Lm*) is a gram-positive food-borne pathogen that causes meningitis and abortion. The ability of *Listeria* to induce its internalization (entry) into human cells is critical for virulence. One major pathway of entry is mediated by interaction of the bacterial surface protein InlA with its host receptor, E-cadherin. Binding of InlA to E-cadherin results in localized polymerization of the host actin cytoskeleton, which contributes to remodeling of the host plasma membrane during entry. A key unanswered question is whether host physiological processes apart from actin polymerization are exploited by *Listeria* in order to promote entry through E-cadherin. Therefore, the objective of this study is to assess the role of other host processes in InlA-mediated entry of *Listeria*. We report that InlA-mediated entry of *Lm* involves bacterial stimulation of the host process of exocytosis. Using the exocytic probe VAMP3-GFP and latex beads coated with InlA as a model for entry of *Lm*, we found entry of beads is accompanied by localized stimulation of exocytosis. Many exocytic pathways are known to involve the exocyst complex, which tethers exocytic vesicles to the plasma membrane prior to membrane fusion. Importantly, we found that RNAi-mediated depletion of components of the exocyst complex (Sec6, Exo70, and Exo84) reduced entry of InlA-coated beads. These results demonstrate a role for the exocyst in InlA-mediated internalization. We also assessed the role of the exocyst regulators RalA, Rab8, and Rab11 using RNAi or dominant negative mutants. Our results show that uptake of InlA-coated beads was reduced upon inhibition of each of these regulators, indicating roles for RalA, Rab8, and Rab11 in entry. Furthermore, we found that n-ethylmaleimide-sensitive factor (NSF), a protein critical for membrane fusion during exocytosis, is required for InlA-mediated entry. These studies were performed using a cell-permeable NSF peptide that inhibits the function of endogenous NSF. Finally, by performing biotinylation of surface proteins, we showed that inhibition of NSF or the exocyst proteins Sec6, Exo70, Exo84 failed to significantly reduce surface levels of E-cadherin. These findings suggest that exocytosis promotes InlA-mediated entry by acting downstream of InlA/E-cadherin interaction. Collectively, our results indicate that *Lm* subverts exocytosis through the host exocyst in order to facilitate entry into human cells.

P2595/B854

The Small Gtp-binding Protein Arf6 Is Recruited to Actin-rich *Listeria Monocytogenes P*Rotrusions during Cell-to-cell Spreading.

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Listeria monocytogenes commander the actin filaments of their host cells to propel themselves throughout the host cytoplasm using actin-rich comet/rocket tails that form behind the advancing bacteria. ADP-ribosylation factor 6 (Arf6), a small Guanine triphosphate (GTP)- binding protein plays a variety of roles during endocytic events and actin-remodeling and has long been known to be involved in the initial invasion of *L. monocytogenes* into non-phagocytic epithelial cells. A key part of the virulence of *L. monocytogenes* is their ability to move from one cell to another. This cell-to-cell spreading involves actin tails pushing the bacteria into the host cell plasma membrane. These membrane protrusions form corresponding invaginations in the neighbouring cells allowing for the uptake of the bacteria into the neighbouring cells. Using immunofluorescence microscopy and Arf6-GFP we demonstrate that Arf6 is present on both the *L. monocytogenes* protrusions and their corresponding invaginations.

P2596/B855

The Host GTPase Arf1 and Its Effectors Ap1 and Pick1 Stimulate Actin Polymerization and Exocytosis to Promote Entry of *Listeria*.

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Listeria monocytogenes is a food-borne bacterium that causes gastroenteritis, meningitis, or abortion. Critical for disease is the ability of *Listeria* to induce its internalization ('entry') into human epithelial cells. One of the major pathways of *Listeria* entry is mediated by binding of the bacterial surface protein InlB to its host receptor the Met receptor tyrosine kinase. InlB/Met interaction stimulates uptake of *Listeria* by inducing two host processes: localized polymerization of the actin cytoskeleton and focal exocytosis. How actin cytoskeletal changes and exocytosis are controlled during entry is not well understood. One group of mammalian proteins that has the potential to control actin filament assembly and exocytosis is Arf family GTPases the objective of this study was to investigate the role of these GTPases in infection of human cells with *Listeria*. Our results indicate an important role for the GTPase Arf1 and its effectors AP1 and PICK1 in actin polymerization and exocytosis during InlB-dependent uptake. Depletion of Arf1 by RNAi or inhibition of Arf1 activity using a dominant negative allele impaired InlB-dependent internalization, indicating an important role for Arf1 in this process. InlB stimulated an increase in the GTP-bound form of Arf1, demonstrating that this bacterial protein activates Arf1. RNAi and immunolocalization studies indicated that Arf1 controls exocytosis and actin polymerization during entry by recruiting the effectors AP1 and PICK1 to the plasma membrane. In turn, AP1 and PICK1 promoted plasma membrane translocation of both Filamin a (FlnA) and Exo70, two host proteins previously found to mediate exocytosis during InlB-dependent internalization. PICK1 mediated recruitment of Exo70, but not FlnA. Collectively these results indicate that Arf1, AP1, and PICK1 stimulate exocytosis by redistributing FlnA and Exo70 to the plasma membrane. We propose that Arf1, AP1, and PICK1 serve as key coordinators of actin polymerization and exocytosis during infection of host cells by *Listeria.*

P2597/B856

Determining the Role of E-cadherin in Promoting *Listeria Monocytogenes* Cell-to-cell Spread in Epithelial Monolayers.

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Listeria monocytogenes is a bacterial pathogen that is able to spread between adjacent cells in an epithelial monolayer without exposure to the extracellular space. While certain bacterial factors that mediate *L. monocytogenes* cell-to-cell spread have been identified, there is little understanding of how host cell factors directly facilitate spread from one cell to the other. The cell junction protein E-cadherin has been shown to strongly enhance cell-to-cell spread for other intracellular pathogens and might play a role in *L. monocytogenes* as well. To study E-cadherin's role in *L. monocytogenes* cell-to-cell spread, we transduced A431D cells, which do not express endogenous E-cadherin, with wild-type E-cadherin or delta cyto E-cadherin that lacks E-cadherin's cytoplasmic domain. Spread efficacy was greater between cells expressing WT E-cadherin than cells expressing delta cyto or no E-cadherin, suggesting that E-cadherin's cytoplasmic domain contributes to spread. Moreover, *L. monocytogenes* spread more efficiently between two neighboring WT E-cad cells than from a WT E-cad donor to a null E-cad recipient

cell. This leads us to conclude that E-cadherin's cytoplasmic domain participates in spread at the recipient side of cell contacts. As EGF enhanced spread in cells expressing WT E-cadherin and not in cells expressing delta cyto E-cadherin, we hypothesized that E-cadherin's contribution to spread might arise from its ability to bind caveolin-1 and undergo endocytosis. In this scenario, donor cell protrusions containing *L. monocytogenes* would be internalized into the recipient cell simultaneous with E-cadherin through a caveolin-dependent process. In support of this hypothesis, we have evidence showing that blocking caveolin-mediated endocytosis with the drug filipin and preventing E-cadherin's interaction with caveolin using the caveolin-scaffolding domain peptide, cavtratin, both reduce *L. monocytogenes* cell-to-cell spread in WT E-cad cells but not delta cyto E-cad cells. We also found that slowing down E-cadherin turnover through an orthogonal method, by expressing a version of E-cadherin that is unable to bind the ubiquitin ligase, hakai, also results in less efficient *L. monocytogenes* cell-to-cell spread. Therefore, caveolin-mediated internalization of E-cadherin is a process used by *L. monocytogenes to* spread between neighboring epithelial cells.

P2598/B857

***L. Pneumophila* Secreted Effector Protein LegC7 Facilitates Fusion of Endosomal and ER-derived Compartments in *S. Cerevisiae*.**

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L. pneumophila is an opportunistic bacterial intracellular pathogen and the causative agent of Legionnaires' disease. Inside human alveolar macrophages, *L. pneumophila* secretes >300 effector proteins into the host cell via a Type IV secretion system, thus allowing *L. pneumophila* to avoid lysosomal degradation and recruit host ER-derived vesicles to the Legionella-containing vacuole (LCV). One such effector protein, LegC7, has been previously shown to disrupt normal endosomal traffic when expressed in yeast, although the basis for this activity was not discovered. In this study, we find that CORVET/HOPS tethering complex mutants suppress the LegC7-induced growth inhibition of yeast and that LegC7 appears to strongly colocalize with Vps8p, suggesting a role for CORVET in LegC7 activity in vivo. In addition, LegC7 colocalizes with Sec63p, induces abnormal ER morphology upon expression, and causes the vacuolar protease-dependent degradation of ER luminal protein Kar2p. These phenotypes are suppressed in *vps33Δ* strains, suggesting a role for CORVET/HOPS in the LegC7-dependent disruption of normal ER physiology. Furthermore, flow cytometry analysis of cells expressing an ER lumen-directed, redox-sensitive GFP reveals that LegC7 expression also induces further oxidation of the ER lumen, and a split-GFP study suggests that mixing of ER-derived compartment with endosomes occurs during LegC7 expression. These data provide strong evidence that LegC7 facilitates the fusion of ER-derived vesicles directly with endosomal compartments in yeast, which implicates LegC7 in the recruitment of ER-derived vesicles to the LCV with the help of host membrane tethering machinery in human alveolar macrophages during a *L. pneumophila* infection.

P2599/B858

Identification of Novel *Legionella*-Resistant Genes in *Dictyostelium*.

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Studies in the social amoeba, *Dictyostelium discoideum*, have led to the identification of a variety of host genes that modulate intracellular growth of several pathogens, including *Legionella pneumophila* and *Mycobacterium marinum.* However, there is a dearth of information regarding molecular mechanisms of host resistance towards these infectious organisms. In this study, we identified several new genes in *Dictyostelium* which confer resistance to infection caused by *L. pneumophila*. To identify resistance-associated genes, we developed a novel 'resistance assay' using the *Legionella*-susceptible wild type and -resistant Nramp1-overexpressing *Dictyostelium* strains as controls. In this assay, at 96 hours post-infection, *Legionella*-infected Nramp1-overexpressing cells could be significantly recovered in liquid culture medium as opposed to infected wild type cells which showed negligible growth. Using this screening assay, we investigated 18 defined, single gene deletion *Dictyostelium* mutants for resistance to *Legionella* infection. Of these, 4 mutants, encompassing defects in phagocytosis, vesicle trafficking, development or chemotaxis, showed 6- to 400-fold increase in resistance to infection, in comparison to wild type cells. Interestingly, resistance to bacterial infection in these mutant strains increased a further 2.5- to 8-fold upon overexpressing the Nramp1 protein in them. These results suggest that aberrations in expression of multiple host genes confer increased host resistance to infection. Encouraged by these results, we are currently attempting to introduce different combinations of these newly identified mutations in *Dictyostelium* for deciphering host mechanisms which provide resistance to *Legionella* and other intracellular bacterial pathogens.

P2600/B859

Characterization of a Ubiquitin-Associated Bacterial Effector Protein.

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Many intracellular bacterial pathogens survive within eukaryotic host cells by establishing and maintaining a replication-permissive membrane-bound compartment. *Legionella pneumophila*, the causative agent of Legionnaires' disease, establishes its intracellular niche within phagocytic cells by using a specialized secretion system to translocate over 350 effector proteins that can manipulate various host cell components. However, the majority of these effector proteins have yet to be characterized. Here we report Lpg2411 as a novel ubiquitin-associated effector that is translocated during late stages of macrophage infection. Secondary structure predictions revealed Lpg2411 has limited homology to a ubiquitin C-terminal hydrolase. When ectopically produced in HeLa cells, mCherry-Lpg2411 extensively co-localizes with ubiquitin and a Co-IP of mCherry-Lpg2411 from HEK293T lysates revealed Lpg2411 interacts with poly-ubiquitinated proteins. Lpg2411 displays a dynamic localization pattern when translocated into macrophages. HA-tagged Lpg2411 was observed on intracellular tubular structures and punctae as well as plasma membrane-blebbing structures and cellular extensions of infected host cells. Cellular events that occur during late infection are not well established, thus further studies on Lpg2411 will reveal insight into host-pathogen dynamics during late infection.

P2601/B860

Isoform-specific Ras Recruitment to Vacuolar Membranes by the Intracellular Pathogen*Legionella Pneumophila*.

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The intracellular bacterial pathogen *Legionella pneumophila* (Lp) is the primary cause of the most common water-borne illness in the US, a severe pneumonia called Legionnaire's disease. Internalized into human host cells by phagocytosis, an Lp bacterium converts the phagocytic vesicle surrounding it during entry into a protective, membrane enclosed, replication compartment called the *Legionella*-containing vacuole (LCV). It achieves this membrane conversion by secreting over 300 bacterial effector proteins into it's host via a type IV secretion system. While characterizing host endolysosomal protein trafficking during Lp infection in live cells, we discovered that the small GTPase NRas, but not its homologs KRas or HRas, is recruited to the *Legionella*-containing vacuole (LCV) by a bacterial effector-dependent mechanism. Once established, NRas recruitment to the LCV was maintained until late times of infection. Ras GTPases are molecular switches that can signal from cellular membranes to regulate a number of fundamental processes involved in growth and survival in all eukaryotes. The essential role of Ras in cell biology makes it a fitting target for pathogens seeking to usurp control of host cell machineries. We sought to define the mechanism and signaling consequences of selective NRas recruitment to the LCV. Human Ras isoforms are targeted to membranes by residues within their C-terminal tails, and differ slightly in their intracellular distribution as well as in their ability to signal on endomembranes. We found the C-terminal tail of NRas was necessary and sufficient for its recruitment to the LCV during infection, requiring both its farnesylation and single palmitoylation site. Interestingly, we could direct HRas recruitment to the LCV by mutating its tail domain to similarly permit farnesylation plus a single palmitoylation. As has been previously reported, this mutation shifts steady state HRas to accumulate more on Golgi membranes compared to wild-type. Selectivity for NRas recruitment to the LCV thus depends on its unique PTM profile, and potentially from the Golgi enrichment afforded by such. A single secreted Lp effector protein, designated Defects in NRas Recruitment (DenR), was found to mediate LCV recruitment of NRas, and partially colocalized on membranes with NRas during ectopic expression in cells. Additionally, both constitutively active NRas as well as an activate-Ras reporter (Halo-Raf-1 Ras-binding-domain) could be detected on LCV throughout infection, suggesting the formation of a presistent NRas signaling compartment. These results provide new insight into the manipulation of Ras GTPases during pathogen infection, and may more broadly offer novel understandings of the differential regulation of Ras protooncogenes in health and disease.

P2602/B861

Discovering the Function of the Phosphoinositide-binding *Legionella* Effector, Lpg0405.

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Phosphoinositide (PI) lipids have proven to be a common target for efficient localization of various pathogens' effector proteins in the host cell. As organelle identity is in part dictated by the species of phosphoinositide present, effectors have acquired PI-binding domains to concentrate at specific locations. *Legionella pneumophila*, the causative agent of the potentially fatal pneumonia termed Legionnaires' disease, has been found to encode a number of effectors that contain these lipid-binding domains that contribute to this pathogen's ability to survive inside human macrophages. *Legionella* avoids degradation by using these effector proteins to prevent the host lysosomes from fusing with the *Legionella*-containing vacuole (LCV). We have recently identified, through an in *vitro* lipid binding assay, that an additional *Legionella* effector, Lpg0405, possesses the ability to bind to PIs. In transiently transfected mammalian cells, mCherry-Lpg0405 showed extensive colocalization with GFP-2xFYVE, a PI(3)P biosensor, and but not GFP-P4Mx2, a PI(4)P biosensor. The lipid-binding domain appears to be

present in the N-terminus of the protein since this fragment, but not the C-terminal fragment, shows membrane localization. Upon depletion of PI(3)P by the PI3-kinase inhibitor, wortmannin, mCherry-Lpg0405's localization became cytosolic. Therefore, PI binding is important for localization of Lpg0405 to cellular membranes. Lpg0405 also showed extensive colocalization with a lysosomal marker, LAMP1. The association with these membrane compartments may hint at a role in preventing lysosomal maturation, an important hallmark of *Legionella* infection. Furthermore, Lpg0405 shows interaction with another *Legionella* effector, Lpg2411 and could be functioning together during infection.

P2603/B862

***Ehrlichia Chaffeensis* Trp120 Interacts with Notch Epidermal Growth Factor Domains to Activate Notch Signaling .**

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Ehrlichia chaffeensis is a small obligately, intracellular gram-negative bacterium, and the etiological agent of human monocytotropic ehrlichiosis (HME), a life-threatening emerging tick-borne zoonosis. Recently, we have shown that the mechanisms whereby *E. chaffeensis* evades host defenses of the macrophage appear to involve activation of Wnt and Notch signaling. Various molecular-based strategies are used by *E. chaffeensis* for intracellular survival, including host-pathogen interactions by secreted tandem repeat protein effectors. Interestingly, TRP120 has been shown to interact with proteins important for activation and regulation of conserved signaling pathways; specifically, Wnt, Notch and Sonic Hedgehog. TRP120 has been shown to directly interact with ADAM17, a Notch metalloprotease, and FBW7, a Notch antagonist. Further, colocalization of TRP120 with both ADAM17 and the Notch-1 receptor has been demonstrated. Ligand-receptor interactions of Notch ligands and Notch-1 occur through direct binding events at epidermal growth factor-like repeats (EGFs) in the extracellular domain (NECD) of the receptor. Importantly the defined ligand binding domain of Notch-1 are EGFs 11-13. Thus, we hypothesize that *E. chaffeensis* TRP120 is a novel non-canonical Notch ligand that activates Notch signaling via tandem repeat amino acids molecularly interacting with NECD-EGF domains 11- 13. We have demonstrated homology of TRP120 and Notch ligands. Specifically, a short motif in TRP120-TR, EDDT, shares homology with Notch ligands. Using surface plasmon resonance, we have determined direct binding of TRP120 and Notch-1 using a Notch-1 recombinant protein containing EGFs 1-13. We have further demonstrated that TRP120-TR and TRP120-NTD are responsible for Notch activation and have currently identified a 32-amino acid motif, inclusive of EDDT, that is required for Notch activation. These results indicate that a specific TRP120-TR sequence is important for the activation of Notch. Understanding the pathobiology of intracellular pathogens may allow for the development of novel therapeutics by targeting host-pathogen protein- protein interactions.

P2604/B863

The Polarity of Epithelial Cells Regulates *Neisseria Gonorrhoeae* Infectivity.

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Neisseria gonorrhoeae (GC) establishes infection in women from the cervix, the gate of the female reproductive tract. To establish infection, GC have to overcome the cervical epithelium. The cervical epithelium is highly heterogeneous, from non-polarized stratified ectocervical epithelial cells to polarized columnar endocervical epithelial cells. Columnar epithelial cells exhibit a polarized distribution of host receptors, the actin cytoskeleton, and form cell-cell junction (the apical junction) supported by

perijunctional actomyosin ring. Stratified epithelial cells display a non-polarized distribution of the actin cytoskeleton and adherens junction. Our previous studies showed that distinct properties of cervical epithelial cells affect GC infectivity in corresponding cervical regions. However, whether the difference in the polarity of cervical epithelial cells, which associates with cell morphology, actin organization, and host receptor distribution, contributes to the differential infectivity of GC in the ectocervix and endocervix is unknown. To address this question, we utilized a non-polarized and polarized epithelial cell model generated from the same cell line on transwells and the human cervical tissue explant model. While GC adhered to both non-polarized and polarized epithelial cells to a similar level, GC invaded into non-polarized epithelial cells more efficiently than into polarized epithelial cells. The expression of phase variable Opa (Opa+) on GC enhanced both GC adhesion and invasion, compared to an Opa deletion mutant (ΔOpa). Inoculation of Opa+ GC induced microvilli elongation in non-polarized epithelial cells, which was concurrent with F-actin recruitment at GC adherent sites. However, we only observed the similar F-actin recruitment in the epithelial cells exfoliated from the ectocervix but not those remained in the ectocervical epithelium. In contrast, inoculation of ΔOpa GC caused a massive disruption of microvilli at the apical surface of polarized epithelial cells, which was concurrent with non-muscle myosin II (NMII)-dependent reduction of F-actin at GC adherent sites of both polarized cell line model and endocervical epithelial cells in tissue explants. GC inoculation only induced intracellular calcium in polarized, but not in non-polarized epithelial cells, which was responsible for NMII activation. Overall, these results suggest that the level of epithelial cell polarity regulates GC infectivity by altering the cytoskeletal and morphological responses of epithelial cells to GC and GC surface molecules.

P2605/B864

Effect of *Helicobacter Pylori O*N the Intercellular Junctions of Pancreatic Cells.

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Helicobacter pylori is a gram-negative bacterium known for its damage to the stomach. However, in recent years it has been detected in other human body parts as pancreas, related to different extragastric diseases. *H. pylori* affects the gastric epithelium by damaging the intercellular junctions (ICJs) with the participation of several virulence factors, allowing the long-term infection. The injury on ICJs could be related to the bacterium' ability for invading other organs. Therefore, the aim of this work is evaluating the effect of *H. pylori* on IJCs of pancreatic cells. By using epithelial cells BxPC-3 derived from human pancreatic ductal adenocarcinoma and as control AGS gastric cells, we evaluated the alterations produced by *H. pylori* infection. The cytopathic effect was measured by retention of methylene blue at different MOI (1:50, 1:100, 1:500 and 1:1000), and after 24 h we observed destruction of pancreatic cell monolayers, similarly as control gastric cells in a MOI-dependent manner, reaching up to 22%. Regarding cellular viability, both cell types decreased up to 30% their viability. Despite the significant morphological changes (11%) produced by *H. pylori* on pancreatic cells, they exhibited a scarce hummingbird phenotype (2%), versus the 63% observed by AGS cells. The permeability of pancreatic cells, was evaluated by TEER measurements and results evidenced a TEER decrease of 30% and 37% at MOI 1:500 after 24 h of pancreatic and gastric cells, respectively. In agreement, the occludin expression diminished in pancreatic cells, suggesting that other ICJ proteins could be also modified. Based on those results we conclude that *H. pylori* is capable to infect pancreatic

cells and produce morphological changes, rearranging the actin cytoskeleton and disrupting the epithelial barrier by injuring ICJs, as is has been demonstrated for gastric cells.

P2606/B865

ALIS Adventures in Macrophage-land: Functional Characterization of Aggresome-like Induced Structures (ALIS) in Response to Infection.

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Recognition of pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) activate the initial immune response in higher eukaryotes. Macrophages and dendritic cells (DCs) serve as sentinels of host defence against foreign invaders. These cells respond to a repertoire of conserved microbial molecules that are recognized by a collection of pattern recognition receptors (PRRs), like Toll-like receptors (TLRs). PAMP recognition by TLRs induces the accumulation of microtubule-associated protein 1A/1B-light chain 3 (LC3) in punctate structures. Macrophage cells form aggresome-like induced structures (ALIS) in response to lipopolysaccharide (LPS) and *E.coli* stimulation via TLR4 signalling. These are detergent-insoluble, non-membranous, ubiquitin, p62/sequestome1 and LC3-positive bodies. In response to infection, immune cells are known to upregulate the p62 mRNA and protein levels. Thus, the formation of ALIS and the presence of p62 in ALIS indicates their role in host defence. To gain insight into the function and composition of ALIS, we are using cell biology, imaging and biochemical approaches. Apart from LPS, we have observed ALIS formation in response to *E. coli* particles*, Bacillus subtilis and Mycobacterium smegmatis*. This indicates that cells sequester these structures via other PRRs as well. Live-imaging microscopy shows the onset of formation of ALIS upon infection. ALIS also appears to associate with vacuoles formed in response to LPS stimulation and the bacteria containing phagophores. Characterization of ALIS using super-resolution structured illumination microscopy (SIM) provides architectural information. We observed that ALIS is an organized complex. This organization is dynamic and changes with the stage of structural maturity. Biochemical enrichment of ALIS was employed to investigate its components by mass spectrometry. Our data indicate the presence of several immune-related proteins. These results suggest that ALIS assembly is a part of the hitherto unknown immune response.

P2607/B866

Genomic Determinants Underlying Rodenticidal Properties of the *Salmonella Enteritidis* Var. Issatschenko.

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Different strains of *Salmonella enterica* can infect a wide range of hosts, including rodents, well-known agricultural pests. The losses from rodents in agriculture comprise up to 28% of the potential yield, which forces to constantly undertake measures against them. Several chemical pesticides (rodenticides) are known to control the numbers of these pests. However, these substances can pose danger to warm-blooded animals and humans. At the end of the 19th century, several strains of *Salmonella enteritidis* with rodent-specific action were isolated by the eminent Russian scientist Boris Issatschenko. Despite the wide usage of bacteria var. Issatschenko at the beginning of the 20th century, there is still no

accurate data on the molecular basis underlying specificity of their virulence factors. These strains represent unique model for studying mechanisms of the host specificity, as they can affect only certain species of the order Rodentia. In the present work we assembled the complete genome of *S. enteritidis* var. Issatschenko strains from the ARRIAM collection using the Nanopore long-read and Illumina short-read sequencing technologies. Having compared the *S. enteritidis* var. Issatschenko genome with other publicly available assemblies of *S. enteritidis* we have found a set of unique single nucleotide polymorphisms in protein-encoding genes of the bacterium Issatschenko. Part of these SNPs is located in coding regions of genes whose protein products are known to be involved in the virulence of *Salmonella*. Summing up, our data indicate that mutations in several genes encoding virulence factors of *Salmonella enteritidis* var. Issatschenko are likely to be responsible for highly specific rodenticidal properties of the. This work was supported by the Russian Science Foundation (Grant No 19-76-00026).

P2608/B867

Internalization of *Borrelia Burgdorferi* into Human Cardiomyocyte AC16 Cells.

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In this study, we tested if *Borrelia burgdorferi* (Bb), an obligate parasitic spirochete that causes Lyme disease, can enter into cardiomyocytes. Approximately 4% of advanced Lyme disease cases exhibit carditis involving an atrioventricular blockade and the development of dilated cardiomyopathy. Bb endocytosed by phagocytic cells are not dependent on serum complement; and proceeds by a non-conical internalization process known as “coiling phagocytosis”. We tested for Bb internalization into several different cell types to determine if penetration is restricted to professional phagocytic cells. Our objective was to determine if entry results from spirochetal penetration, or as a consequence of host-cell phagocytosis. We tracked the entry of spirochetes into the human cardiomyocyte AC16 and mouse fibroblasts 3T3 cell lines by confocal microscopy using a recombinant Bb (B31-strain) engineered to express green fluorescent protein (GFP-Bb). Internalization studies were performed on cells adherent to coverslips. Cells were incubated in RPMI1640 plus 5% FCS at 37^{o}C in 5% CO_2 for 60 min. Cover slips were washed with ice cold PBS three times, fixed with 1% PFA, washed again, and stained with DAPI to label the nuclei. Labeled cells were inspected for internalized GFP-Bb spirochetes. These experiments were replicated in parallel using RAW 264.7 macrophage and JAWSII dendritic cells to demonstrate the processing of intracellular GFP-Bb by professional phagocytic cells. Cell surface labeling used anti-CD44 for AC16 and 3T3 cells, anti-CD86 for macrophage, and anti-CD11c for dendritic cells to demarcate intracellular regions. The fluorescence from GFP-Bb was identified at the cell surface and within internal compartments showing various stages of internalization. Incubation of the cells at 4^{o}C blocks internalization, and incubation at 16^{o}C permits a lower rate of internalization but halts intracellular membrane fusion events. A second experimental series was performed to monitor the processing of internalized GFP-Bb by professional phagocytic cells. After 60 min, cells with internalized GFP-Bb were washed with ice cold media to remove unbound motile extracellular spirochetes and either returned to 37^{o}C to sustain processing of internalized spirochetal cargo, or 16^{o}C to prevent phagosomal hydrolysis. The appearance of fluorescence was detectable following the 60 min incubation at 37^{o}C that was comparable to 16^{o}C, suggesting that low-pH associated processes within the host phagolysosomal compartments were compromised by Bb. These results support the model of intracellular parasites modulating intracellular functions to acquire a “safe-harbor” within host compartments, providing insight into a putative cellular mechanism for persistent borreliosis.

P2609/B868

Subversion of Host Endocytic Trafficking Pathway by the *Legionella Pneumophila* Effector Protein an kx.

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The intracellular bacterium *Legionella pneumophila* proliferates within amoebae and human alveolar macrophages, and it is the causative agent of Legionnaires' disease, a life-threatening pneumonia. During infection, *L. pneumophila* remains enclosed in a membrane-derived vacuole, *Legionella*-containing vacuole (LCV) and survives by translocating effector proteins into the host cytosol. Several of these effectors manipulate Rab GTPases to disrupt vesicular trafficking pathways. The *Legionella* effector protein an kX is a phosphocholine transferases that covalently modifies Rab35 and facilitates avoidance of pathogen degradation. We hypothesize that an kX manipulates host endocytic trafficking pathways to prevent fusion of the LCV with endocytic compartments, to avoid degradation via phagosome maturation. We determined an kX's subcellular localization to the plasma membrane and tubular membrane compartments in mammalian cells by using superresolution microscopy and immunogold transmission electron microscopy. We observed that an kX colocalizes with Rab35, a Rab GTPase in charge to regulate the recycling of cargo back to the plasma membrane in the fast recycling pathway. We quantified the levels of an kX's disruption of endocytic trafficking by using the DQ Red BSA assay. Furthermore, we determined an kX's localization after translocation during infection of amoeba, *Legionella's* natural host. Finally, we investigate an kX's mechanism to disrupt endocytic trafficking and help prevent degradation in amoeba.

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Blood Cells and Vessels

P2610/B870

Studying Pericyte-endothelial Interaction during an giogenesis in Medium Throughput Microfluidic Culture Platform.

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The transition from 2D to 3D cell culture and co-cultures of more relevant cell types are the first steps towards more physiological relevant models in *vitro*. Previously we showed that a standardized microfluidic 3D tissue culture platform, called OrganoPlate® can be used to generate precisely controlled gradients, pump-free and in medium throughput (n=40) for growing blood vessels and inducing controlled 3D angiogenic sprouting [1]. Here we integrated pericytes into the vascular model to look at cell-cell interaction during angiogenesis. Endothelial cells are grown against an extracellular matrix gel and form a vessel/tubule due to the flow. Pericytes are seeded in the ECM or co-seeded with the endothelial cells. The blood vessel is exposed to angiogenic compounds to direct sprouting into the ECM gel. When pericytes were co-seeded with endothelial cells sprouts with a perfusable lumen were formed. In contrast pericytes in the ECM inhibited sprouting. Pericytes in the parental vessel also started to express high levels of α-sma. This model can be used as an in *vitro* screening platform to unravel the important drivers in angiogenesis and vasculogenesis and the involvement of pericytes in these processes. REFERENCES: [1] van Duinen V , Zhu D, Ramakers C, van Zonneveld AJ, Vulto P, Hankemeier T.

Perfused 3D angiogenic sprouting in a high-throughput in vitro platform. An giogenesis. 2019 Feb;22(1):157-165.

P2611/B871

Profilin1 Is an Important Regulator of Developmental and Pathological Retinal Neovascularization.

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Angiogenesis is an essential process of neovascularization during development and tissue repair. However, aberrant angiogenesis exacerbates many diseases including proliferative diabetic retinopathy (PDR), a leading cause of blindness worldwide. Our bioinformatics analyses revealed that actin-binding protein Profilin1 (Pfn1), an important regulator of actin cytoskeleton, is transcriptionally upregulated in vascular endothelial cells (EC) derived from retina of PDR patients prompting us to explore the role of Pfn1 in developmental and pathological angiogenesis in the retina. To this end, we generated for the first time an inducible vascular EC-selective Pfn1 knockout mouse model to demonstrate that post-natal deletion of the *Pfn1* gene in EC leads to prominent reduction in endothelial F-actin abundance, vascular density and branching during developmental angiogenesis in the retina. By computationally guided biochemical screen, we further identified novel small molecule antagonists of Pfn1:actin interaction that are non-cytotoxic, inhibit EC migration and proliferation (key features of angiogenesis) and exhibit anti-antigenic activity on various types of vascular EC, regardless of their tissue of origin, in *vitro*, *ex vivo* and in *vivo. To* model pathological neovascularization in the retina as occurs in PDR, we subjected newborn mice to oxygen-induced retinopathy (OIR) where hyperoxic-to-normoxic transition (mimics a hypoxic shift) elicits strong VEGF-driven pathological neovascular response in the retina. We provide evidence for transcriptional upregulation of Pfn1 in the OIR retina, and further demonstrate that small molecule intervention of Pfn1:actin interaction through intravitreal administration attenuates pathological retinal neovascularization. In summary, these findings provide the first direct evidence of Pfn1's indispensable role in mammalian developmental angiogenesis in *vivo* and a proof-of-concept of susceptibility of pathological angiogenesis to small molecule intervention of Pfn1, laying a conceptual foundation for targeting Pfn1 in angiogenesis-dependent diseases.

P2612/B872

The Concept of Personalized Cell-mediated Gene Therapy Using Gene Modified Leucoconcentrate (GML) Prepared from the Patient's Peripheral Blood.

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Previously we have shown that umbilical cord blood mononuclear cells transduced with adenoviral vectors carrying recombinant human genes is the promising therapeutic approach for cell-mediated gene therapy. Herein for the first time we propose the usage of the peripheral blood leucoconcentrate as a cell system for temporary production of the recombinant biologically active molecules for correction of certain pathological conditions. The mini-pig's peripheral blood was collected into the plastic blood bag. Preparation of the leucoconcentrate and transduction of WBC with chimeric

adenoviral vector (Ad5/35) carrying reporter green fluorescent protein (GFP) gene was performed in the plastic bag. After transduction for 12 hours gene modified leucoconcentrate (GML) was washed out with saline and the plastic bag was kept at room temperature before usage. At the next day after blood donation the mini-pigs were underwent to spinal cord injury and four hours after surgery were injected intravenously with GML. A week after surgery fluorescent microscopy study of the spinal cord sections at the site of injury and the white pulp of spleen revealed WBC expressing GFP. Thus the efficacy of transduction, migratory potential and secretory activity of WBC suggest their usage for temporary production of the specific recombinant biologically active molecules for pathogenetic therapy of the varied nosological form, such as trauma, ischemic, degenerative, autoimmune, infection and other diseases. Results of this study present a simple, safe and effective approach for personalized ex vivo gene therapy based on autoinfusion of GML. This study was supported by the grant of Russian Science Foundation No 16-15-00010.

P2613/B873

The Role of Transglutaminase 2-ROS Vicious Cycle in Hyperglycemic Memory-mediated Vascular Dysfunction in Diabetic Aorta.

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Clinical trials suggest that hyperglycemic memory (HGM) is defined as the long-term diabetic vascular complications even after glycemic control in Type 1 and 2 diabetes ; However, the mechanisms underlying the cellular HGM induced by high glucose remain unclear. We hypothesized that a vicious cycle between transglutaminase 2 (TGase2) and intracellular reactive oxygen species (ROS) plays a key role in HGM-induced vascular dysfunction. We found that hyperglycemia induced persistent oxidative stress, expression of inflammatory adhesion molecules, and apoptosis in the aortic endothelium of HGM mice whose blood glucose levels had been normalized by insulin supplementation. TGase2 activation and ROS generation were in a vicious cycle in the aortic endothelium of HGM mice and also in human aortic endothelial cells after glucose normalization, which played a key role in the sustained expression of inflammatory adhesion molecules and apoptosis. Our findings suggest that the TGase2-ROS vicious cycle plays an important role in HGM-induced endothelial dysfunction.

P2614/B874

Regulation of Endothelial-derived *Pseudomonas Aeruginosa*-induced Secreted Beta Amyloid by Cystatin C.

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Patients who survive hospital-acquired (nosocomial) pneumonia have elevated death rates in the first year following hospital discharge and exhibit additional deficiencies, including decreased memory function. These observations suggest that long-lived deleterious molecules may be produced during the initial infection. Using *Pseudomonas aeruginosa* as a model for nosocomial pneumonia, we demonstrated that infection of pulmonary microvascular endothelial cells (PMVECs) by *P. Aeruginosa* led to the release of cytotoxic oligomeric tau and beta amyloid by the PMVECs. Attempts to deplete the cytotoxins by ultracentrifugation demonstrated an initial increase in cytotoxic activity (one hour of

centrifugation at 150,000 x g) followed by a rapid depletion of cell killing activity at longer periods of centrifugation. Likewise, immuno-depletion of beta amyloid caused increased cytotoxic activity in the residual supernatant. Collectively, these results suggested that cytoprotective forms of beta amyloid, in addition to cytotoxic amyloids, are released during infection processes. To identify possible modulators of beta amyloid activity, mass spectrometry was performed of proteins secreted from PMVECs following infection by *P. Aeruginosa*. These analyses identified three potential regulators of beta amyloid in the secreted material, including gelsolin, clusterin, and cystatin C. Each of these proteins was immuno-depleted individually from bacterial-induced PMVEC secreted supernatant, and the depleted supernatants were added to cultured cells. No change in cytotoxic behavior was observed following depletion of either clusterin or gelsolin. However, supernatants depleted of cystatin C exhibited increased cytotoxic behavior demonstrating that secreted cystatin C has a protective effect. Subsequently, co-immunoprecipitation of cystatin C and beta amyloid was demonstrated. Moreover, cystatin C and beta amyloid pelleted during the first hour of centrifugation at 150,000 x g. Collectively, these data demonstrate that cystatin C is a negative regulator of beta amyloid-induced cytotoxicity following *P. Aeruginosa* infection and identify cystatin C as a potential target for intervention to alleviate the long-term consequences of nosocomial infection.

P2615/B875

Dissecting the Roles of Notch Receptors in Human Haematopoietic Stem Cells.

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Haematopoietic stem cells (HSCs) reside at the top of the haematopoietic cells hierarchy and have the highest self-renewal and differentiation capacities, giving rise to more committed progenitors (HPCs) that become progressively more restricted in terms of their differentiation and self-renewal capacities. The Notch signalling pathway has been linked to cell fate decisions in haematopoiesis, although its role in the regulation of steady-state human HSCs is still largely unexplored. Activation of Notch signalling in *vitro* has been shown to promote self-renewal of HSPCs and has been explored for protocols of *ex vivo* expansion. Nonetheless, a more comprehensive study is necessary to clearly identify the functions of the different Notch receptors in HSPCs regulation.The expression of Notch receptors, NOTCH1 (N1) and NOTCH2 (N2), and their respective activation in UCB HSPCs were quantified at protein level. Both N1 and N2 receptors were found to be highly expressed on HSPC and HSC populations, with higher activation in HSCs. The roles of N1 and N2 receptors were evaluated through lentiviral shRNA-mediated gene silencing in UCB HSPCs, with average knockdown efficiencies of 72% and 89% (at the protein level) achieved for N1 and N2 receptor, respectively. In a long-term culture model to detect HSCs and immature progenitors, we observed that the number of primitive cells was not affected by either knockdown. However, N2 receptor silencing promoted a decrease in HSCs frequency while with N1 receptor silencing only an increase in immature progenitors was observed. In accordance, in a xenotransplantation assay we observed that, although the total human engraftment was not largely affected in primary transplants, N1 and N2 receptor silencing in the transplanted HSPCs promoted different phenotypic outcomes in *vivo*. N1 receptor silencing favoured myeloid over lymphoid differentiation and increased frequency of haematopoietic progenitors but had no effect in HSCs, whereas N2 receptor silencing significantly decreased HSCs frequency, had no effect in progenitor populations and a milder effect on differentiation.These results suggest that, although extracellular N1

and N2 receptors are both highly expressed on HSCs, activation of N2 receptor has a more determinant role in the fate of HSCs than N1 receptor and the respective genetic programs are under evaluation.

P2616/B876

Heparin Blocks Foam Cell Formation and Reverse Cholesterol Transport.

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[Objective] Heparin is a potent drug to inhibit blood coagulation. The property of this drug, it contains a lot of negative charged residue. To clear the effect of negative charged material against foarm cell formation and reduce proceeding atherosclerosis, foam cell formation and reverse cholesterol transport with /without Heparin , several PPAR agonists, ARB and vascular statins can be examined as positive and negative effertors to ABCA1 transcription and translocation **[Method]** LDL and sdLDL were prepared from human plasma by KBr density gradient separation. AcLDL and OxLDL were prepared by Acetic anhydrate treatment , CuSO4 oxidation respectively. Foam cells were prepared by J774 cells incubating with these modified LDL for 24-72hr, and cultured up to 3-6 days in the presence of LPDS +/- HDL and Heparin, PPAR agonists, ARB or statin. FC(Free cholesterol) rich lipid droplets were detected by Filipin staining. **[Results & Discussion]** FC-rich Lipid droplets were detected by Filipin staining in these modified LDL induced foam cells. Foam cell formation reduced markedly in the presence of Heparin. HDL, a potent material to remove FC from foam cell via ABCA1, ABCG7 pathway, did not affect to reverse cholesterol transport in heparin treated foam cells. However, Vascular statin molecules (Simvastatin, Atorvastatin) were well known as a potent activator of ABCA1 translocation in foam cells, also reduced FC-rich lipid droplet formation, and increase ABCA1 expression level in AcLDL, OxLDL induced foam cells. These materials did not recover FC translocation activity to HDL in Heparin treated foam cells. Considering FC rich lipid droplet formation, ARB molecules (Angiotensin II AT1 receptors blockers; Losartan, Irbesartan, Telmisartan) had a potent activity as well as PPAR alfa, gamma agonists, and reduce FC-rich lipid droplet formation, However, in the presence of Heparin, ARB did not affect on this activity. These significantly relationship of Heparin against AcLDL induced foam cells, among the FC-rich lipid droplet formation, reverse cholesterol transport and foam cell death may reflect to development of atherosclerosis correlated in IHD patients. Contact me by; mmori@cis.ac.jp

P2617/B877

Exogenous Mitochondria Regulate Intrinsic Apoptosis Pathway and Oxphos Function in Acute Hindlimb Ischemia.

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Mitochondria, the critical role of survival and death in mammalian cells, are involved in such as calcium homeostasis, biogenesis, and regulation of apoptosis, reactive oxygen species production. As ischemic damage has been reported to be involved in mitochondrial dysfunction, new treatment of transplanting exogenous mitochondria into ischemic heart tissue before ischemic reperfusion have recently been reported. However, the therapeutic mechanism of transplanted mitochondria is still controversial. In this study, we determined whether transplanted mitochondria contribute to angiogenesis and oxidative phosphorylation (OXPHOS) via inhibition of the apoptosis pathway in acute ischemic hindlimb model. One week after mitochondria transplantation, blood flow was assessed by laser Doppler imaging. Ischemic limb significantly increased blood flow as well as skeletal muscle mass. The ischemic muscles

were harvested for detection of mitochondrial apoptotic markers and mitochondrial OXPHOS proteins. Consequently, the expression of OXPHOS protein was increased in the group injected with exogenous mitochondria. On the other hand, all expressions of Bcl-2, Bax, cytochrome c, caspase 3 and cleaved caspase 3 were decreased compared to the sham control group. Based on our results, new findings revealed that transplanted mitochondria inhibit cellular apoptosis and promote angiogenesis.

P2618/B878

Stromal Cells Promote Neovascular Invasion Across Tissue Interfaces.

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Rapid vascularization is essential for the survival of implanted and repairing tissues. Via angiogenesis, neovessels grow through tissue interfaces and create a network that is fully integrated with the existing microvasculature. Understanding the mechanisms that guide vessels across the interface between two tissues is essential for effectively supporting tissue vascularization. Towards this, we used an experimental tissue interface model involving a “core” plug of collagen gel embedded in a “field” of fresh collagen, resulting in two distinct regions separated by a thin, high-density layer of collagen as determined by second harmonic generation imaging (SHG) and scanning electron microscopy (SEM). In this model, we incorporate whole isolated microvessel fragments (intact capillaries, arterioles, venules) isolated from adipose tissue. We have previously shown that microvessels spontaneously sprout, grow, and connect when embedded in collagen. To model growth across the interface, microvessels were incorporated into the core region with or without stromal cells. Crossing events, where angiogenic neovessels grew across the interface, were counted, then normalized to both interface length and overall microvessel density. Microvessel density was quantified from images taken using a GE in cell 6500 confocal scanner. Without the additional stromal cells, neovessels grow and sprout within the core, but rarely cross this high-density interface; instead growing parallel to collagen fibrils of the interface. When stromal cells were incorporated into the core, neovessels crossed the interface and began to populate the field. When stromal cells were instead incorporated into the field (with microvessels in the core), even more crossings were recorded. SEM imaging indicated that crossing events do not grossly alter or degrade the interface structure. In the presence of a VEGF-A trap, neovessel crossing events were significantly reduced. However, VEGF-A alone was insufficient to stimulate crossing, suggesting a stromal cell-mediated, but VEGF-A dependent mechanism. Finally, we determined that macrophages are necessary for this process. Microvessels were incorporated with the complete stromal cell fraction or with stromal cells lacking CD11b/c^{+}. An increase in crossings was visible only when CD11b/c^{+} cells were present. This indicates that stromal cells, particularly tissue-resident macrophages, play a critical role in guiding neovessel growth during angiogenesis across tissue interfaces. We propose that these cells create a spatiotemporal gradient of VEGF, guiding neovessels across the interface. This suggests that inclusion of macrophages within a fabricated tissue would promote engraftment following implantation and accelerate graft perfusion

P2619/B879

Lysyl Oxidase and MMP12 Directionally Regulate Age-associated Arterial Stiffening.

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Arterial stiffening is a hallmark of aging and a risk factor for cardiovascular disease (CVD). We have previously shown that arterial stiffening in mice develops between 12 to 24 months of age. In the premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS), patients display arterial stiffness at an early age, and typically die in their teenage years as a consequence of CVD. Here we have compared the process of arterial stiffening in normal and premature aging using carotid arteries isolated from WT and HGPS (LMNA608G/608G) mice. Pressure myography showed that carotid arteries of 2-month HGPS mice stiffened prematurely, with a circumferential stiffness similar to 24-month WT controls. Transmission electron microscopy (TEM) revealed increased amounts of collagen within the elastin folds of both 2-month HGPS and 24-month WT carotid arteries relative to 2-month WT controls. Additionally, immunostaining and RT-qPCR revealed increased expression of Lysyl oxidase (LOX) in young HGPS arteries, and an incremental increase of arterial LOX expression at 12-24 months in WT arteries. Moreover, treatment of HGPS mice with the pan-Lox inhibitor β-aminopropionitrile (BAPN) restored near-normal circumferential arterial mechanics to HGPS carotid arteries. LOX covalently crosslinks the newly synthesized fibrillar collagens and elastin to regulate their tensile strength, but since new elastin synthesis ends early in life, the LOX effect on arterial stiffness is probably restricted to collagen. Although similarly stiff circumferentially, the arteries of 24-month WT mice differed from young HGPS arteries in two ways. They displayed additional stiffening in the axial direction, and this correlated with a high prevalence of elastin breakage not seen in HGPS or the 2-month WT controls. Previous work in our lab showed that the abundance of MMP12, a potent elastase, increases as arteries stiffen with age. The deletion of MMP12 in mice significantly reduced the axial stiffening seen in normal aging whereas the circumferential stiffening of these arteries persisted. The arteries from 24-month MMP12 null mice also had a significant reduction in elastin breakage when compared to age-matched WT controls. These results associate age-dependent circumferential stiffening with Lox-mediated collagen crosslinking and axial stiffening with MMP12-mediated elastin degradation. Supported by NIH grants AG047373 and AG062140 and NSF agreement CMMI 1548571.

P2620/B880

Role of Liver Dysfunction in Blood Brain Barrier and Consequences to Alzheimers Disease.

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Alzheimers disease (AD) is the most common cause of dementia in older-aged people. During the last decade, it has become been clear that the synaptic loss and neuronal degeneration underlying cognitive impairments are caused by small aggregates termed Abeta oligomers. One key pathway that may underlie Abeta accumulation is dysfunction of the blood brain barrier (BBB). The BBB is a layer of tightly packed cells that regulates the flow of molecules between the brain and blood. There is evidence of BBB impairment and dysfunction in AD research models and patients. It remains unclear whether Abeta oligomer clearance through the BBB is affected by this impairment. The liver is a key organ mediating the clearance of Abeta from the circulation; thus, dysfunction of these organs can impair Abeta

clearance. Liver dysfunction occurs in multiple contexts, including nonalcoholic fatty liver disease (NAFLD), a common condition that is associated with obesity and inflammation. We hypothesize that NAFLD can intensify the AD-related BBB impairment to exacerbate AD pathology. The aim of our research was to evaluate the impact of NAFLD in AD pathology, specifically in Abeta-oligomer dynamics, BBB integrity, and LRP-1 levels. We used mice fed with a high fat diet as models of NAFLD/NASH. AD pathology was determined through histological staining with Thioflavin S, and Aβ-specific and GFAP antibodies. BBB integrity was followed by penetration studies and immunofluorescence for tight junctions proteins, and LRP-1 levels was determined by immunohistochemistry. We found that Abeta plaques and Abeta oligomers are increased in brain of NAFLD/NASH mice, while levels of occluding and ZO1 are decreased in BBB. LRP1 levels were reduced in liver of NAFLD/NASH affected mice. Altogether our results indicated that liver damage in the context of NAFLD/NASH, impairs liver Abeta clearance, and affects BBB integrity, with the involvement of LRP1. We anticipate NAFLD will exacerbate contribute to a vicious cycle that perpetuates the imbalance in peripheral Aβ-oligomer clearance as a result of liver dysfunction and chronic inflammation. Funding: CIBQA, Depto. Ciencias Quimicas y Biologicas. Universidad Bernardo OHiggins. Laboratorio de Hepatologia experimental. CARE ChileUC PFB 12/2007. P. Universidad Catolica de Chile.

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Human Peripheral Blood Mononuclear Cell Expression of Stimulated by Retinoic Acid 6.

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Coined as the anti-infective vitamin, vitamin a has pleiotropic roles in immunity. Vitamin a deficiency results in impaired innate immunity and skewed adaptive immune responses towards inflammatory T helper type one responses at the expense of T helper type two responses. The objective of this study was to characterize the expression of the receptor for holo-retinol binding protein, stimulated by retinoic acid 6 (STRA6), which facilitates cellular retinol uptake. Holo-retinol binding protein also activates STRA6-dependent Janus kinase (JAK) 2 and signal transducer and activator of transcription (STAT) 5 signaling with eventual transcriptional up-regulation of suppressor of cytokine signaling (SOCS) 3. Multicolor flow cytometry and confocal microscopy was used to determine expression of STRA6 on human peripheral blood mononuclear cells. Expression of STRA6 was determined by median fluorescence intensity of STRA6 in B lymphocytes, T lymphocyte subsets, natural killer cell subsets, monocyte subsets, and dendritic cell subsets. Monocytes expressed significantly higher STRA6 than any mononuclear subset and B lymphocytes expressed significantly lower STRA6 than any mononuclear cell subset. T helper cells (CD4+) expressed significantly higher STRA6 than T cytotoxic cells (CD8+). The CD14-dim/CD16+ monocyte subset expressed significantly higher STRA6 than CD14+/CD16- and CD14+/CD16+ monocytes. There was no significant difference in STRA6 expression on natural killer cell subsets or dendritic cell subsets. The cell line retinal pigmented epithelial - 19 (ARPE-19) cells expressed high amounts of STRA6. However, confocal microscopy showed punctate intracellular staining of STRA6 in ARPE-19 cells instead of plasma membrane-associated staining. It is unknown if STRA6 is expressed on the plasma membrane or intracellularly in human peripheral blood mononuclear cells. Determining cellular localization of STRA6 in peripheral blood mononuclear cells is a future direction as well as mechanisms of regulation of STRA6 expression. These data provide a characterization of STRA6 expression by human immune cells which provide baseline data to investigate cellular uptake of vitamin A, as well as JAK-STAT-SOCS axis, in inflammatory and homeostatic conditions. The implications of the

data are an understanding of the vitamin a transport and STRA6 signaling in relation to immune cell energy metabolism as the JAK-STAT-SOCS signaling can reduce insulin sensitivity.

P2622/B882

Effect of BIA 10-2474 in Salt-loaded Stroke-prone Spontaneously Hypertensive Rats.

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In 2016 one person died and four others had mild-to-severe neurological symptoms during a phase I clinical trial of the fatty acid amide hydrolase inhibitor BIA 10-2474, with reports that the subject who died, after receiving the 50 mg dose for 5 days in the clinical trial, showed evidence of severe brain microhemorrhage and several of the surviving subjects also showed evidence of mild to moderate microhemorrhage. To determine if BIA 10-2474 might contribute to cerebral blood vessels rupturing, the effects of the compound were explored in stroke-prone spontaneously hypertensive rats (SHRSP) by evaluating mortality rate and examining cerebrovascular damage.Male SHRSP were 60-day-old at the start of the study. Ten SHRSP received standard rodent diet and regular tap water (control group). The remaining 60 rats were placed on a stroke-prone rodent diet and given 1% NaCl to drink. Rats on the stroke-prone diet and high-salt were divided into two groups receiving either vehicle (0.2% HPMC) or BIA 10-2474 (30 mg/kg p.o.). Treatments were administered daily until approximately half the rats in either the vehicle or BIA 10-2474 group had died or euthanized for ethical reasons. At the end of the study, surviving animals were euthanized and brains were removed, fixed and embedded; seven cutting levels were taken from the brains, whenever possible, and stained by H&E. Histological changes were described according to distribution, severity and morphologic character. Lesion area was measured, whenever possible, for hemorrhage, edema, inflammation and necrosis.The study was terminated after 45 days when 53.3% (16/30) of the animals receiving vehicle and 43.3% (13/30) of animals receiving BIA 10-2474 had died. No deaths were recorded in the control group. Log-rank test revealed no effect of BIA 10-2474 treatment on mortality rate compared with vehicle in animals on stroke-prone diet and high-salt intake (P=0.1882). In those rats surviving at the end of the study, histological lesions consisted of hemorrhage, edema and inflammation; necrosis was not observed. In rats on high-salt intake and receiving vehicle or BIA 10-2474, the type of lesion, location, number and size was similar comparing affected animals. Daily treatment with BIA 10-2474 at the dose of 30 mg/kg did not affect mortality rate in this model and, in surviving animals, did not increase the incidence of hemorrhage or edema.

Exocrine and Endocrine Organs

P2623/B883

Effect of Human Placenta-derived Mesenchymal Stem Cells on Antioxidant Factors of Ovary in Taa-injured Rat Model.

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Ovary is a very important organ for female reproductive system as well as health. Reactive oxygen species (ROS) are generated as by-products during ovarian physiological metabolism and their unbalance between ROS production and clearance induce ovarian dysfunction such as infertility, polycystic ovarian syndrome (PCOS) and early menopause. In previous reports, placenta-derived mesenchymal stem cells (PD-MSCs) promotes folliculogenesis in ovariectomized rat model. However, there is no evidence that placenta-derived stem cells (PD-MSCs) have can preserve ovary function of ovarian dysfunction model via antioxidant effect. So, the objectives of the present study are to analyze the effect of human placenta-derived mesenchymal stem cells on the antioxidant factors in the ovary of thioacetamide (TAA) injured rat models by measuring folliculogenesis factors. In vivo experiment was designed by injecting 300mg/kg of TAA every week for 12 weeks in 7-week-old female Sprague-Dawley rats. Human PD-MSC (PD-MSCs Tx) and saline (Non-Tx) injected at week 8 via tail vein. Explant culture experiment was designed by culturing a half-cut ovary in a 24-well-plate treated with 70mM of TAA for 24 hours. First treatment group received $1x10^4$ PD-MSCs and second treatment group received $5x10^4$ PD-MSCs per each well for 24 hours. Ovaries were collected in each experiments and analyzed for ROS and antioxidant factors (NOX4, CAT, HO-1, SOD), folliculogenesis factors (Lhx8, Lin28a, Nanos3, Nobox) and E2 levels. Expression of antioxidant factors increased and ROS factors significantly decreased in in vivo ovaries after PD-MSC Tx. ($p<0.05$). Ex vivo ovaries showed enhancement in antioxidant activities correlated with amount of PD-MSCs co-cultivated. E2 production level in ex vivo ovaries increased as amount of PD-MSCs co-cultivated increased compared to control group. Both samples showed increase in folliculogenesis factors in RNA levels after PD-MSCs co-cultivtion. Based on these results, PD-MSC Tx effectively enhances ovarian function in TAA-treated rat models by regulating ROS and antioxidant factors and increases folliculogenesis activity. These findings lead to the potential of new therapeutic strategies of PD-MSCs in reproductive medicine. **Funding Source**: This research was supported by a grant from the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning, Republic of Korea (grant number : NRF-2017M3A9B406)

P2624/B884

The Constitutive Formation of USP8-STAM1 Complex Plays an Important Role in the Onset of Cushing's Disease.

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Ubiquitin-specific protease 8 (USP8) deubiquitinates and stabilizes some receptors for growth factors and hormones, thereby regulating the receptor-mediated signaling. USP8 interacts with 14-3-3 proteins. We had reported that missense mutations of the 14-3-3 binding motif in USP8 very frequently occur in pituitary tumors in Cushing’s disease. In this disease, pituitary tumors secret excess

adrenocorticotrophic hormone (ACTH), leading to the onset of hypercortisolism symptoms. Here we show the pathogenic mechanisms by which USP8 mutation leads to the onset of Cushing's disease. We focused on STAM1, one of USP8 binding proteins. STAM1 harbors ubiquitin-binding domains and can help USP8 to recognize substrate proteins. Firstly, we analyzed USP8-STAM1 interaction in HEK293 cells. Wild-type USP8 ($USP8^{wt}$) interacted with STAM1 in an EGF-dependent manner, whereas the Cushing's disease-associated mutant USP8 ($USP8^{mut}$) constitutively interacted with STAM1. USP8 harbors three putative STAM binding motifs (SBMs), and we found that the region aa 738-747 (SBM3) in USP8 interacts with STAM1. Interestingly, the SBM3 is in close proximity to the 14-3-3 binding motif (aa 715-720). Overexpression of difopein, a peptide that inhibits the interaction of 14-3-3 and the binding proteins, increased the interaction of $USP8^{wt}$ with STAM1. It indicates that the dissociation of 14-3-3 may make the SBM3 available to STAM1. We tried to identify substrate protein(s) of $USP8^{mut}$-STAM1 complex. The vasopressin V1b receptor is a GPCR that is specifically expressed in pituitary corticotrophs, and mediates vasopressin-induced ACTH secretion. We found that $USP8^{mut}$-STAM1 complex binds to ER-residing immature V1b. The treatment with proteasome inhibitors increased immature V1b, indicating that immature V1b is unstable and degraded through the ubiquitin-proteasome pathway under basal condition. Overexpression of $USP8^{mut}$ reduced ubiquitinated V1b. Overexpression of $USP8^{mut}$, not $USP8^{wt}$, increased immature V1b as well as mature V1b. The increase in V1b was inhibited by amino acid substitution of the SBM3 in $USP8^{mut}$, confirming the importance of $USP8^{mut}$-STAM1 interaction. Finally, we observed that overexpression of $USP8^{mut}$, not $USP8^{wt}$, enhanced vasopressin-induced inositol phosphate production. Taking these data together, we conclude that 1) the Cushing's disease associated mutation of USP8 leads to the constitutive formation of $USP8^{mut}$-STAM1 complex; 2) $USP8^{mut}$-STAM1 complex binds to and deubiquitinates immature V1b, thereby preventing its degradation; 3) through these mechanisms, $USP8^{mut}$-STAM1 complex increases cell surface V1b levels and vasopressin signaling, possibly leading to excess ACTH secretion and the onset of Cushing's disease.

P2625/B885

Cortactin Deficiency Causes Pancreatic Epithelial Dysfunction.

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Cortactin is a multifaceted actin-binding protein containing several domains allowing for interaction with other proteins, thus enabling cortactin to fulfill essential roles in diverse actin-related cellular processes including adhesion and migration. Moreover, homeostasis maintenance of the intestinal barrier is regulated by cortactin; since in *cortactin*-KO mice colon epithelial permeability is increased and expression and localization of intercellular junction (ICJs) proteins is altered. However, it remains unknown whether absence of cortactin causes also damage in other epithelia such as in the pancreas. Therefore, the aim of this work is to analyze the effect of cortactin deficiency on pancreatic IJCs proteins, as well as on epithelial permeability of a cortactin-depleted ductal pancreatic cell line.Histologically, the pancreas of KO-mice looks apparently normal compared with those from wild-type animals. However, western blot and immunofluorescence analyses of pancreatic tissue revealed that cortactin-deficiency provoked a decrease of filamentous actin, and reduced expression of the IJC proteins occludin, claudin-1 and ZO-1. Moreover, these IJS relocalized from cellular borders to the cytosol in the absence of cortactin. By contrast, only a slight decrease of E-cadherin was detected, with similar location in WT and KO tissues. To investigate the effect of cortactin on pancreatic epithelial permeability, cortactin-depleted BxPC-3 cells were generated using shRNA and their transepithelial

electrical resistance (TEER) was monitored. Cortactin-KD cells exhibited 45% lower TEER than control cells. Accordingly, ICJs proteins were also less expressed and mis-localized.In conclusion, our results demonstrate that in the pancreas, cortactin also participates in the regulation of proper IJC architecture and epithelial permeability. Thus, cortactin seems to be a critical regulator of epithelial homeostasis in many organs.

P2626/B886

Haemoglobin Expression in Prostate Cells: Hypoxia and Dht Signalling?

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Haemoglobins are expressed in erythroid cells. We found haemoglobin expression in prostate cells and aimed at characterizing whether haemoglobin expression in prostate cells is due to hypoxia or DHT signalling. *HBA1* and *HBE* were found in 2D cultured RWPE1, and its expression increased in 3D cultured RWPE1, regardless of the presence/absence of dihydrotestosterone (DHT). In 2D cultured cells HBA was found in the nucleus. In 3D, however, the lack of DHT changed HBA subcellular localization: cells in the center of the spheroid presented higher amounts of HBA, localized mainly on the cytoplasm, whereas cells in the periphery presented the same nuclear localization as 2D cells. DHT treatment abolished HBA cytoplasmic localization. Interestingly, 2D cells cultured under hypoxia had increased *HBA2*, *HBA1* and *HBE* expression (51-, 119- and 450-fold, respectively) compared to 2D cells not submitted to hypoxia. $CoCl_2$ treatment also increased *HBA2*, *HBA1* and *HBE* expression, (9-, 7- and 16-fold, respectively). *HBA2*, *HBA1* and *HBE* are expressed in RWPE1 cells, and their expression increase in 3D culture. Spheroids untreated with DHT presented both cytoplasmic (center cells) and nuclear (peripheric cells) localization of HBA. **Financial support:** FAPESP

P2627/B887

Murine Model of Chronic Prostatitis Involves the Mtor Pathway.

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Patients with chronic prostatitis/chronic pelvic pain syndrome (CP/CPPS) suffer from chronic pelvic pain. Surveys conducted by the National Institute of Health (NIH) reported a significant impact on the quality of life of CP/CPPS patients with chronic symptoms due to few viable therapeutic targets. Studies using fMRI showed that various cortices in the brain (e.g., prelimbic, anterior cingulate cortex, and insular cortex) are altered in patients diagnosed with CP/CPPS after several years. Interestingly, a strong link between mTOR signaling in the anterior cingulate cortex and the development of neuropathic pain has emerged in several rodent models. Therefore, we seek to examine whether the mTOR pathway plays a key role in anterior cingulate cortex sensory processing in mice with chronic pelvic pain in a model of CP/CPPS called experimental autoimmune prostatitis (EAP). Our experiments were performed on male C57BL/6J (B6) mice (6-8 weeks old). To elicit EAP, the experimental group received a subcutaneous injection of rat prostate antigen (1 mg/ml). We conducted behavioral pain testing (via von Frey filaments) on all mice to assess the development of pelvic tactile allodynia on days 0, 7, 14, 20, and 30. On day 30, mice were perfused and the anterior cingulate cortex and spinal cord lumbosacral region were excised from mice with EAP and respective control cohorts. The anterior cingulate cortex and spinal cord were processed for either immunofluorescence or immunoblots. Our behavioral measurements verified that mice with EAP develop pelvic pain at day 7 thru day 30. Moreover, the immunofluorescence and immunoblot data showed that the lumbosacral region of the spinal cord

collected from mice with EAP had increased phosphorylation of ribosomal S6 (S40/244), a substrate site for S6K1 and downstream of the mTOR cascade, in neurons compared to control cohorts at day 30. Similarly, the anterior cingulate cortex from B6 with EAP showed elevated levels of phosphorylated S6 (240/244) proteins compared to control. Altogether, this study interconnects the development of chronic pelvic pain to the activation of the mTOR signaling cascade in regions of the spinal cord and brain relevant to CP/CPPS. More importantly, the data presented in this study suggests that the mTOR pathway at specific brain sites may signal neuroplastic changes in higher-order neurons that hinder the long-term effectiveness of current treatment options.

P2628/B888

Targeting MT1-MMP: Therapeutic Opportunities in Obesity, Type 2 Diabetes and Other Associated Diseases.

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Obesity and diabetes are major causes of morbidity and mortality. Obesity is known to be the main risk factor for various non-communicable diseases, in particular type 2 diabetes. There is an ongoing need to identify non-invasive therapeutic approaches for the management of obese patients with type 2 diabetes to achieve their glycemic and weight loss goals.Recently, mutations in MT1-MMP (MMP14), a membrane bound metalloproteinase responsible for extracellular matrix remodeling and pericellular proteolysis, have been associated with human obese and diabetic traits. In this talk, the role of MT1-MMP in energy and glucose homeostasis and the molecular mechanism by which MT1-MMP regulates body weight and insulin sensitivity will be discussed. MT1-MMP contributes to the development of metabolic disorders through increasing energy intake and impairing insulin sensitivity. We found that MT1-MMP is a suppressor for GDF15-GFRAL signaling pathway, a newly identified central nervous system circuit that regulates metabolism and food intake in response to stress. In addition, MT1-MMP also impairs insulin signaling in the control of peripheral insulin sensitivity and glucose metabolism. These results indicate that MT1-MMP constitutes an important modulator of insulin sensitivity and energy homeostasis. Thus, targeting MT1-MMP represents potential therapeutics for the management of obesity and type 2 diabetes, two diseases with a need for combined treatment strategies.

P2629/B889

Investigating Abnormal Gene Expression in Zebrafish Ciliopathy Models.

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Polycystic kidney disease (PKD) is a medically important disorder that is characterized by the formation of multiple epithelium-lined, liquid-filled cysts in the kidney. Affecting one in 1000 live births, autosomal dominant polycystic kidney disease (ADPKD) is among the most common monogenetic disorders in humans (Gabow and Grantham, 1997). Over-proliferation of renal epithelial cells contributes to PKD pathogenesis (Nadasdy et al., 1995). Moreover, the cilium is thought to play a central role in the etiology of PKD (Ong and Wheatley, 2003). However, the precise role of cilia in PKD pathogenesis is not well understood. In previous studies, we identified zebrafish mutants of *arl13b*, a cilia biogenesis gene, and *pkd2*, a causal gene of ADPKD. To identify downstream targets of ciliary and Polycystin defects, we performed transcriptome analysis using RNA-seq and identified hundreds of genes with changed expression levels. To validate the RNA-seq results, we performed qPCR for four of the hits that are

changed in both mutants. We utilized *pkd2 and arl13b* genes as the internal comparisons between both mutants to ensure experiment accuracy. As expected, our qPCR results showed that *pkd2* mRNA level is decreased in *pkd2* mutants, while *arl13b* mRNA level is decreased in *arl13b* mutants. Moreover *tcap and ntd5* RNA are significantly elevated in both mutants, while *glis3 and klf4* RNA is significantly elevated in *arl13b* mutants. We hope to expand our analysis in the future to fully validate the RNA-seq results and to perform pathway analysis to identify key signaling events affected by ciliary and Polycystin defects.

P2630/B890

Molecular Mechanisms of Prolactin in the Scented Glands of the Male Muskrats (*Ondatra Zibethicus*).

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Prolactin (PRL) production in mammals has been demonstrated in extrapituitary gland, which can activate autocrine/paracrine signaling pathways to regulate physiological activity. The muskrat (*Ondatra zibethicus*) is a medium-sized semiaquatic rodent that is native to North America and has been introduced to China since 1957. The muskrat is a seasonal breeder with sexually active period of about 8 months from March to October. The muskrat has a pair of scented glands near its tail that give off a musky odor to attract females. The object of our study is to investigate the expression of prolactin (PRL), prolactin receptor (PRLR) and signal transducers and activators of transcription 5 (STAT5) in the scented glands of the breeding and non-breeding seasons and to determine the levels of prolactin (PRL) in the sera and scented glands of the muskrats. We found that the weight and volume of the scented glands in the breeding season were significantly higher than those of the non-breeding season. Immunohistochemical data showed that PRL, PRLR and STAT5 were found in the glandular cells and epithelial cells of the scented glands in both the seasons. Furthermore, we found that PRL, PRLR and STAT5 had higher immunoreactivities during the breeding season when compared to those of the non-breeding season. With parallel expressions of PRL, PRLR and STAT5 at the protein and mRNA levels were significantly higher in the scented gland during the breeding season than those of during the non-breeding season. The concentrations of PRL in scented glandular tissues and sera were measured by enzyme-linked immunosorbent assay (ELISA), and their levels were both notably higher in the breeding season than those of in the non-breeding season. These findings suggested that the scented glands of the muskrats were capable of extrapituitary synthesis of PRL, which may attribute PRL to an endocrine or autocrine/paracrine mediator. The current study showed that the scented glands of the muskrats were capable of extrapituitary synthesis of PRL which may attribute PRL a specific function as an endocrine or autocrine/paracrine mediator.

Digestive and Excretory Organs

P2631/B891

Analysis of the Impact of P21 Dependent G2 Cell Cycle Arrest on Renal Fibrosis.

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The progression of renal fibrosis is correlated with kidney function. Although several processes are implicated in the regulation of renal fibrosis, including the epithelial-mesenchymal transition (EMT) and cell cycle arrest, the underlying molecular mechanisms are largely unknown. Here, by using in vivo and

in vitro models, we have examined the impact of renal tubular cell-cycle arrest on the progression of renal fibrosis. To induce renal fibrosis in mice, we have conducted unilateral urinary obstruction (UUO) into male mice. We have found the proximal tubular cells arrested at the G2 of the cell cycle under the obstructed condition, especially in the early stage of the damage where fibrotic features was not seen clearly. Interestingly, the G2 arrest partially dependents on p21, which is a cyclin dependent kinase (CDK) inhibitor. The newly constructed monoclonal antibody against p21 revealed that the protein levels of p21 were sharply upregulated in response to the damage during the initial stage, but dropped down toward the later stage. To investigate the requirement of p21 for the progression of renal fibrosis, we constructed the novel *p21* deficient mice by *i*-GONAD method. Compared with wild-type mice, *p21* deficient mice showed the exacerbation of the fibrosis. Thus, the G2 arrest in early stage of the damage have a protective role in the progression of renal fibrosis.

P2632/B892

Extracellular Vesicles from Senescent Cholangiocytes Differentially Affect Target Cells Via Epidermal Growth Factor Receptor Activation.

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Primary Sclerosing Cholangitis (PSC) is a chronic, cholestatic liver disease of unknown etiology characterized by peribiliary inflammation and fibrosis. We reported that cholangiocyte senescence (a state of cell cycle arrest, apoptosis resistance, and hypersecretion of bioactive molecules) is a prominent feature of PSC. Extracellular vesicles (EVs) have emerged as prominent mediators of cell signaling. Thus, we tested the hypothesis that EVs from senescent cholangiocytes have a unique proteomic profile and influence biliary and peribiliary target cell phenotype. EVs were isolated by ultracentrifugation from cultured normal human cholangiocytes (NHCs), cultured cholangiocytes from PSC patients, and NHCs experimentally induced to senescence with LPS (donor cells). NHCs, malignant human cholangiocytes (MHCs), and monocytes (target cells) were exposed to 10^8 EVs from each donor cell population and assessed for proliferation, MAPK activation, and migration. Additionally, we isolated EVs from plasma of wild-type and $Mdr2^{-/-}$ mice (a murine model of PSC), and assessed mouse monocyte migration and activation. EVs exhibited the size (40-150nm) and protein markers (CD63) of exosomes. The number of EVs released from senescent human cholangiocytes (both experimentally induced or PSC patient derived) was increased (~6-fold); similarly, the EVs in plasma from $Mdr2^{-/-}$ mice were increased (~2-fold). Additionally, EVs from senescent human cholangiocytes were enriched (~2-fold) in multiple growth factors, including EGF, as assessed by growth factor array. NHCs exposed to EVs from senescent human cholangiocytes showed increased NRas and ERK1/2 activation (~4 and ~8-fold, respectively). Moreover, EVs from senescent human cholangiocytes promoted proliferation (~40% increase) of NHCs and MHCs, findings that were blocked by Erlotinib, an EGF receptor inhibitor. Furthermore, EVs from senescent human cholangiocytes induced EGF-dependent Interleukin 1-beta and Tumor necrosis factor-alpha expression (~4-fold) and migration (~2-fold) of human monocytes (THP-1); similarly, $Mdr2^{-/-}$ mouse plasma EVs induced activation (~4-fold) of mouse monocytes. In summary, senescent human cholangiocytes, both PSC-derived and LPS- induced, secrete increased numbers of EVs with the characteristics of exosomes. These EVs are enriched in growth factors, including EGFR ligands, and mediate EGF-dependent cellular responses in target cells. The data continue to support the importance of cholangiocyte senescence in PSC pathogenesis, directly implicate EVs in cholangiocyte proliferation, malignant progression, and immune cell activation and migration, and identify novel therapeutics approaches for PSC.

P2633/B893

Clearance Or Prevention of P16ink4a-associated Cholangiocyte Senescence Improves Inflammation and Fibrosis in the Mdr2-/- Mouse Model of Primary Sclerosing Cholangitis.

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Primary sclerosing cholangitis (PSC) is an idiopathic, progressive disease of the bile ducts. We reported that biliary epithelial cell (cholangiocyte) senescence, characterized by cell cycle arrest and apoptosis resistance, is important in PSC pathogenesis. Moreover, we demonstrated that the cyclin dependent kinase inhibitor, CDKN2A (p16), a central mediator of senescence, is increased in cholangiocytes of PSC patients and in a PSC mouse model ($mdr2^{-/-}$). Here, we use genetic approaches to determine whether targeted removal or genetic deletion of p16 positive cells improves biochemical markers of liver injury, portal fibrosis and inflammation in the $mdr2^{-/-}$ mouse. We employed two novel genetic murine models to assess the role of p16 in the pathophysiology of PSC. The INK-ATTAC mouse allows for the selective apoptotic removal of p16 expressing cells following administration of the dimerizing molecule, AP20187. Hence, we generated double homozygous INK-ATTAC X $mdr2^{-/-}$ mice and treated the experimental group with AP20187. Additionally, we generated a mouse deficient in both $p16^{-/-}$ and $mdr2^{-/-}$ ($p16^{-/-}$ X $mdr2^{-/-}$ mouse). Following AP20187 treatment (INK-ATTAC X $mdr2^{-/-}$) or aging ($p16^{-/-}$ X $mdr2^{-/-}$), we assessed severity of portal inflammation and hepatic fibrosis via hydroxyproline assays and siruis-red staining of liver sections. We also measured monocyte chemoattractant protein-1 (mcp-1), a key regulator of macrophage infiltration, and senescence markers, p16 and p21, by real-time PCR in rodent livers. In the AP20187 treated INK-ATTAC X $mdr2^{-/-}$ mice, fibrosis decreased by 50%, mcp-1 was reduced by 60%, and p16 and p21 were reduced by ~35% and ~70%, respectively. In the $p16^{-/-}$ X $mdr2^{-/-}$ mice, fibrosis was reduced by 25-50%, and Mcp-1 and p21 were each reduced by 90%. We have now shown in two novel genetic mouse models of PSC that removal or prevention of cholangiocyte senescence improves portal fibrosis and inflammation. Together our data continue to support an important pathophysiologic role of senescence in the progression of PSC and suggest that the targeted removal of senescent cholangiocytes (senolytic therapy) is an attractive therapeutic approach.

P2634/B894

Anti-cancer Potential Of*Fagonia Indica*Against Hepatocellular Carcinoma through Expression Regulation of Cytochrome P450 Pathway.

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Background: Hepatocellular carcinoma is one of the most frequent death causing cancers worldwide. The incidence of HCC is growing at an exponential rate in Pakistan with more cases seen in male gender. HCC involves multiple alterations in large number of molecular pathways that lead into cancer progression and malignancy. Objectives: Present study aimed to investigate the anti-cancer effects of medicinal plant *Fagonia indica*against hepatocellular carcinoma. In addition, the potential molecular mechanisms regulated were also analyzed. Methodology: Liver carcinoma cell line, Huh7 was used. $IC_{50}$was determined by using different concentrations of plant extract and comparison with control. Cell Cytotoxicity analysis and DNA fragmentation were performed. PCR was performed for the analysis of regulation of cytochrome P450 pathway. Results: Cell cytotoxicity assay showed significant reduction in cell viability with even low doses of plant extract. DNA fragmentation and down regulation of cancer

markers further confirmed the anti-cancer effects of the plant. On the other hand, regulation of various genes of cytochrome P450 depicted an involvement of the pathway in occurrence and cure of hepatocellular carcinoma. In conclusion, with further detailed experiments, current study proposes *Fagonia indica*as a promising anti-cancer drug against hepatocellular carcinoma.

P2635/B895

Anti-fibrotic and Pro-angiogenic Effects of WKYMVm Peptide in a Rat Model of Bile Duct Ligation.

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Hepatic failure, chronic liver injury, is one of the diseases related to difficult-to-cure liver diseases and it has shown high morbidity and mortality. The chronic liver disease involves inflammation, activation of hepatic stellate cells (HSCs) resulting in fibrogenesis, necrosis of hepatocytes caused by vascular blockade. Fibrogenesis in hepatic failure impairs vasculature of the liver simultaneously dysfunction of liver sinusoidal endothelial cells (LSECs). WKYMVm peptide, which is composed of Trp-Lys-Tyr-Met-Val-D-Met, is a strong agonist of formyl peptide receptor 2 (FPR2). Recently, WKYMVm peptide has been reported as the therapeutic factor in several diseases such as ischemic and wound healing models through migration and proliferation of angiogenic cells. However, the effects of WKYMVm peptide in chronic liver disease have not been elucidated. Therefore, our objectives of this study were to evaluate the condition of hepatic fibrosis and vascularization and analyze the expression of key factors related to hepatic fibrosis and angiogenesis through treatment of WKYMVm peptide in bile duct ligation (BDL) rat model and in *vitro* model. Collagen accumulation using Sirius Red staining in rat liver tissues of BDL was alleviated in WKYMVm treated groups compared with non-treated group (p<0.05). Also, the fibrotic markers (Collagen I and α-SMA) were significantly down-regulated in WKYMVm treated groups versus non-treated groups (p<0.05). While the hepatic fibrosis was drastically declined, the mRNA level of angiogenic factors such as VEGF and Endoglin were dramatically increased in WKYMVm treated groups (p<0.05). In *vitro* model damaged by transforming growth factor-β (TGF-β) in rat HSCs, the area of α-SMA in cytoplasm were significantly decreased in WKYMVm peptide treated group by immunofluorescence (p<0.05). Furthermore, WKYMVm peptide significantly up-regulated hepatic function through hepatocyte proliferation and albumin expression (p<0.05). These findings suggest that WKYMVm peptide mitigates hepatic fibrosis and regenerates damaged vasculatures. Therefore, the WKYMVm peptide has therapeutic potential for anti-fibrosis and hepatic regeneration. This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (HI17C1050020017) and the National Research Foundation of Korea Grant funded by the Korean Government (F19SN22T1305).

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Human Placenta-derived Mesenchymal Stem Cells Enhance Hepatocyte Regenerationvia Antioxidant Effect in Taa Injured Rat Model.

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Mesenchymal stem cells (MSCs) has been a new trend of cell therapy in regenerative medicine, including liver disease. Liver fibrosis is induced by imbalance between reactive oxygen species (ROS) and antioxidant factors, and lead to irreversible stage, cirrhosis. However, there is no evidence that placenta-derived stem cells (PD-MSCs) have can reverse early stage of liver cirrhosis model via antioxidant and hepatocyte regeneration effect. We therefore compared the expression of reactive oxygen species factor and antioxidant factor in TAA-injured rat model in which PD-MSC was transplanted. Additionally, we verified the hepatocyte regenerative function of the stem cell by measuring the albumin expression. In serum level, liver damage marker aspartate aminotransferase (AST), Alanine aminotransferase(ALT) and bilirubin increased in TAA-injured model and showed a significant decrease in PD-MSC treated model ($p<0.05$). On other hand, albumin and Nox4 the liver regeneration marker, decreased in TAA treated model and showed a significant increase in PD-MSC treated model ($p<0.05$). The expression of genes involved in antioxidant factors such as SOD1 and catalase in TAA-injured rat liver were increased in PD-MSC transplanted group. The dominant antioxidant factor which determines the final step in antioxidation, catalase, shows an equitable pattern in which decreases in TAA treated model and increases in PD-MSC transplanted model. These findings lead to the potential of the therapeutic effect of PD-MSCs in liver cirrhosis.

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Regional Calcium Dynamics in *Drosophila* Adult Midgut.

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Although calcium dynamics are widely studied in cell culture, little has been reported on the topic for adult organs. We find striking regional and cell-type specific differences in calcium dynamics in the three major cell types of the adult *Drosophila* intestine. The adult fruit fly intestine is a compelling platform for studying intercellular dynamics and interactions. This stem cell-based epithelium is a reductionist model system of the mammalian small bowel with conserved features. The multi-cellular epithelium consists of three major cell types: progenitor cells that give rise to new cells (including stem cells), enteroendocrine cells that are hormone signaling cells, and enterocytes that are large absorptive cells. We find regional and cell-type specific variations in calcium signaling dynamics within this functionally compartmentalized organ, using confocal imaging of *ex vivo* cultured fly intestines. Progenitor and enteroendocrine cells indicate oscillatory intracellular calcium dynamics of slower and faster frequencies respectively, and enterocytes demonstrate the presence of traveling calcium waves. Our findings may establish a novel model for studying spatio-temporal organization of cellular to organ-level calcium dynamics in a renewable adult organ.

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Factors That Dysregulate Pannexin 1 Channel Expression in the Diabetic Bladder Urothelium.

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Pannexin 1 (Panx1) channels are essential components of the urothelial mechanosensory-transduction system and play important roles in sensation of bladder distention. Our studies with diabetic mice have shown that urothelial Panx1 expression changes with disease progression and correlates with bladder transition from a compensated overactive to decompensated underactive state. Hyperglycemia and polyuria are viewed as main factors leading to emergence and temporal progression of diabetic bladder dysfunction (DBD). Moreover, tissue hypoxia caused by polyuria-induced bladder overdistension is also proposed as a factor in DBD. However, the individual contribution of hyperglycemia, polyuria and hypoxia to DBD has yet to be determined. Our objective is to evaluate the role played by each of these factors in dysregulating Panx1 expression in urothelium. In this study, we used immortalized human and mouse urothelial cell cultures that were submitted to three conditions: (1) High extracellular glucose (HG; 450 mg/dL glucose) or control glucose level (100 mg/dL) for 8, 12, 24 hours, 4 and 8 days; (2) hypoxia (1% oxygen) or normoxia (21% oxygen) for 1, 4, 6 and 8 hours, and (3) mechanical stimulation by cyclic uniaxial stretch (5 min at 10% strain with 5 min rest intervals) for 2 and 5 hours or non-stimulated. Total RNA was extracted from these cells and processed for Panx1 mRNA analysis by qRT-PCR. Short-term exposure to HG increased Panx1 expression (12 hours: 1.20±0.03; N=3; $p<0.05$), whereas prolonged exposure downregulated Panx1 expression (8 days: 0.83±0.04; N=3; $p<0.01$) relative to controls. Exposure to hypoxic conditions induced a progressive increase in Panx1 expression (8 hours: 1.63±0.21; N=3; $p<0.01$) relative to controls. Prolonged cyclic-stretch downregulated Panx1 expression (5 hours: 0.66±0.07; N=3; $p<0.05$) relative to non-stimulated controls. These findings indicate that each of the factors normally encountered in the diabetic bladder environment, i.e. high extracellular glucose levels, prolonged polyuria-induced mechanical stimulation and hypoxia, can individually contribute to dysregulate Panx1 expression in the bladder urothelium and thereby, impair urothelial function and bladder sensation in diabetes.

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Serum Gamma-Glutamyl Transpeptidase in Normal Physiology and Pathology.

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The level of the enzyme gamma-glutamyl transpeptidase (GGT, aka gamma-glutamyl transferase) in the serum has long been used clinically as marker of liver disease. The role of GGT in tissues is well known including its role in cysteine homeostasis. But what role, if any, GGT activity in the serum plays is unclear. GGT is a cell surface enzyme anchored to the membrane by a single transmembrane domain. The mechanism by which it is released from the cell membrane and enters the serum has not been determined. The objective of this study was to investigate differences between the serum versus tissue GGT. We examined the data for insight into the possible roles of serum GGT activity in the progression of liver disease. In humans, serum GGT activity of 0 to 60 U/L is considered within the normal limits. We examined GGT in bile and in serum from healthy patients and those with liver disease. The GGT in bile was observed as a smear at the top of a native polyacrylamide gel suggesting a hydrophobic form of the

enzyme with the transmembrane domain intact. Treatment of the sample with papain, which cleaves the transmembrane portion of the GGT protein, yielded a form of GGT that ran as a single sharp band in a native polyacrylamide gel, indicating a single hydrophilic form of the enzyme. The very low level of GGT in serum from healthy individuals migrates within the gels predominantly as a single hydrophilic form. GGT in the serum of patients with hepatitis or hepatocellular carcinoma migrates as multiple bands and smears revealing a mixture of hydrophobic and hydrophilic forms. High serum GGT is associated with cholestasis suggesting that in many liver disorders GGT on the bile canalicular membrane of the hepatocytes is solubilized by bile acids. Our data indicate that high levels of GGT activity in serum can reveal a heptobiliary pathology, but the activity is unlikely to contribute to the disease. Consistent with our conclusion is a recent report of two families with mutations in GGT. The mutations eliminate the transmembrane domain of the enzyme. The activity of GGT in the serum is very high but these individuals have no liver or other pathology, further indicating that highly elevated levels of serum GGT can identify liver disorders, though this biomarker is not specific and does not play a role in liver pathology.

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Novel Insights into the Crosstalk between Mineralocorticoid Receptor and Extracellular Vesicles in Kupffer Cells Activation in Nash.

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Patients with Non-alcoholic steatohepatitis (NASH) exhibit increased local cortisol production and portal hypercortisolism and a dysregulation of the mineralocorticoid receptor (MR) expression(1,2). Notably, MR has recently been identified as a regulator of macrophage polarization. Kupffer cells (KCs), liver resident macrophages, undergo fundamental changes during the development of NASH and are critical in initiating liver damage and inflammation(3,4). The accumulation and inflammatory polarization (M1) of hepatic macrophages is considered a hallmark feature of progressive disease in NASH patients(5). The purpose of this study was to assess whether MR inhibition specifically in KC prevents M1 polarization and thus ameliorates NASH progression. The role of the MR was studied in myeloid cell-targeted MR knockout mice (MyMRKO)(6). Three-month-old animals were fed with methionine and choline deficient (MCD) diet as experimental NASH model, for 3 weeks. Livers were weighed, fixed and stained with hematoxilin/eosin and oil red. NAS score system was used. As an in *vitro* approach, a primary culture of Kupffer cell was treated with EVs isolated from steatotic hepatocytes and Aldosterone. MR modulation was assessed using eplerenone and siRNA transfection. Pro-inflammatory response was evaluated via real-time PCR (IL-1b, CD68, CD206, CD80, TNF-α, IL4, IL6). Our results showed that in a murine NASH model myeloid-specific deletion of MR ameliorated inflammatory foci induction and steatosis in MyMRKO+MCD compared to WT+MCD. EVs and Aldosterone induced KC M1 polarization, since the expression of IL-1b and CD68 were increased after EVs and Aldosterone incubation. In contrast, the induction of KCs M1 polarization was prevented in MyMRKO KCs. In addition, we found that aldosterone, synergized with EVs in the activation of KCs and this effect was abolished in MyMRKO. Our results indicate that MR contributes to KCs activation and M1 polarization in the development of the inflammatory response in NASH progression in a mechanism that involves a crosstalk between EVs derived from steatotic Hepatocytes and Aldosterone.This work was partially funded by grants from the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 1191145 to MA, FONDECYT 11171001 to DC), the Center for Aging and Regeneration [CARE PFB12/2007 (M.A.), and the grant from Millennium Institute on Immunology and Immunotherapy (No P09/016-F to AK).